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STUDIES OF THE DISTRIBUTION OF CARBON DIOXIDE BETWEEN CELLS AND PLASMA.*

By L. W. SMITH, J. H. MEANS, and M. N. WOODWELL.

(From the Medical Service of the Massachusetts General Hospital, Boston.)

(Received for publication, November 9, 1920.)

The manner in which CO_2 is transported from the tissues to the lungs by the blood has recently been the subject of considerable discussion (1–3). To throw further light on this sufficiently

CORRECTION.

On page 215, Vol. XLIII, No. 1, August, 1920, lines 20 and 21, the formula $3\ (NH_4)_2O \cdot P_2O_5 \cdot 17\ MoO_3 \cdot MO_2 + H_2O$ should read $3\ (NH_4)_2O \cdot P_2O_5 \cdot 17\ MoO_3 \cdot MoO_2 + n\ H_2O$. The formulas on page 216 of the same volume should be similarly corrected.

made in duplicate.

At the same time the proportion of cells to plasma in each sample was determined by means of the hematocrit, and the relative amounts of CO_2 in the cells and in the plasma of a unit of blood were calculated therefrom. For example if the CO_2 content of a sample of whole blood is 50 volumes per cent and if that of the plasma of the same sample is 62 volumes per cent

* This paper is Study No. 7 in a series of studies of the physiology and pathology of the blood from the Harvard Medical School and allied hospitals. The expenses were in part defrayed by the Proctor Fund. A preliminary report on this work was read at the Annual Meeting of the American Society for Clinical Investigation, Atlantic City, New Jersey, May 3, 1920.

and if the hematocrit shows that the proportion of cells to plasma in that sample is as 40 : 60, then in 100 volumes of whole blood 37.2 volumes will be contained in the plasma thus

$$\frac{60}{100} \times 62 = 37.2$$

and 12.8 volumes will be contained in the cells thus

$$50 - 37.2 = 12.8$$

All the hematocrit observations were made in duplicate.

Findings in Normal Persons.

We first made a study of the distribution of CO_2 between the cells and plasma of normal venous blood. The average results are shown in Table I. The average CO_2 content of eighteen

TABLE I.

Average Figures for Venous Blood.

Series.	CO_2 in whole blood.	CO_2 in plasma.	CO_2 in plasma of a unit of blood.	CO_2 in cells of a unit of blood.
	vol. per cent	*vol. per cent*	*vol. per cent*	*vol. per cent*
Normal. 18 bloods from 13 subjects.				
Maximum	62	64	40.8	26.1
Average	57	61	36	21
Minimum	53	58	32.5	15.4
Anemia. 14 bloods from 14 patients.				
Maximum	64	66	60.0	10
Average	58	60	53	5
Minimum	48	48	42.2	1.4

samples of venous blood from thirteen normal persons was 57 volumes of CO_2 per 100 volumes of whole blood and, of these 57 volumes of CO_2, 36 volumes were in the plasma and 21 in the cells.

We next made a study of the differences in CO_2 distribution in arterial and venous blood. For this purpose samples of blood were drawn within a few minutes of each other from the arm vein and the radial artery. The results of these observations are given in Table II.

The average CO_2 content of eight normal arterial whole bloods was 50.4 volumes per cent and of the venous 58.7 volumes per cent. The parallel findings on the plasma were arterial 61.0 and venous 62.5 volumes per cent. These findings are similar to those in the literature. Thus Harrop (6) in ten persons with normal hearts and lungs found an average of 49.7 volumes per cent of CO_2 in the arterial and 54.7 volumes per cent in the venous blood. Peters and Barr (7) in three normal subjects found an average of 50.5 volumes per cent in the arterial and 59.1 volumes per cent in the venous whole blood. Our normal venous-arterial whole blood difference thus was 8.3, Harrop's 5.0, and that of Peters and Barr 8.6 volumes per cent.

A series of arterial and venous plasma CO_2 contents has been published by Stadie and Van Slyke (8). The average figures for thirty-one samples from twenty-one patients[1] were arterial plasma 60.4 and venous plasma 63.6 volumes per cent. These observations were chiefly on pneumonia patients with arterial anoxemia of varying grades, but some were on normal persons. Even so their figures are quite like our normal plasma figures, though their venous-arterial plasma difference is 3.2 volumes per cent as against ours of 1.5 volumes per cent. It is not shown in their table which are the normal subjects, but if we take those who had an arterial oxygen unsaturation of less than 8 volumes per cent we find the average venous plasma CO_2 content to be 67.0 and the arterial 63.5 volumes per cent making the venous-arterial plasma difference 3.5 volumes per cent.

When on the basis of the hematocrit observations we study the distribution of the CO_2 content of arterial and venous whole blood between the cells and the plasma, we get the interesting averages of 35.8 volumes of CO_2 in the plasma of 100 volumes of arterial blood, and 36.5 volumes in that of venous blood. At the same time the cells of 100 volumes of arterial blood contain on an average 14.6 volumes of CO_2 while those of venous blood contain 22.2 volumes.

In other words as blood passes from artery to vein, it gains on an average 8.3 volumes per cent of CO_2, but of this less than 1 volume per cent, 0.7 volume per cent to be exact, is gained by

[1] In these averages we have taken from their table only cases in which arterial and venous determinations were made.

TABLE II.

Observations on Arterial and Venous Blood.

Subject.	Observed.								Calculated.						Remarks.
	Hematocrit.				CO_2				CO_2				CO_2 of V plasma minus CO_2 of A plasma.	CO_2 of V cells minus CO_2 of A cells.	
	Cells.		Plasma.		Whole blood.		Plasma.		Plasma of a unit of whole blood.		Cells of a unit of whole blood.				
	A*	V*	A	V	A	V	A	V	A	V	A	V			
	per cent	*per cent*	*per cent*	*per cent*	*vol. per cent*	*vol. per cent*	*vol. per cent*	*vol. per cent*	*vol. per cent*	*vol. per cent*	*vol. per cent*	*vol. per cent*	*vol. per cent*	*vol. per cent*	
Dr. S.	42	42	58	58	50	56	62	62	35.9	35.9	14.1	20.1	0.0	6.0	Normal subject.
Miss M.	37	39	63	61	46	56	60	62	37.8	37.8	8.2	18.2	0.0	10.0	" "
Dr. M.	45	43	55	57	54	60	62	63	34.1	35.9	19.9	24.1	1.8	4.2	" "
" B.	44	44	56	56	48	56	58	60	32.4	33.6	15.6	22.4	1.2	6.8	" "
" S.	40	40	60	60	50	58	62	63	37.2	37.8	12.8	20.2	0.6	7.4	" "
Mr. A.	38	39	62	61	49	60	62	64	38.4	39.0	10.6	21.0	0.6	10.4	Surgical patient. Fracture; otherwise normal.
" B.	41	42	59	58	52	62	60	62	35.4	35.9	16.6	26.1	0.5	9.5	" " "
" C.	43	43	57	57	54	62	62	64	35.3	36.5	18.7	25.5	1.2	6.8	" " "
Normal average.	41.2	41.5	58.7	58.5	50.4	58.7	61.0	62.5	**35.8**	**36.5**	**14.6**	**22.2**	**0.7**	**7.6**	

Mrs. S.	11	11	89	89	56	60	60	62	53.4	55.1	2.6	4.9	1.7	2.3	Primary anemia. Hemoglobin 40 per cent. Red blood count 1.0 million.
" C.	13	13	87	87	52	56	58	58	50.4	50.4	1.6	5.6	0.0	4.0	Secondary anemia. Hemoglobin 52 per cent. Red blood count 4.0 millions.
Mr. B.	14	14	86	86	52	56	59	60	50.7	51.6	1.3	4.4	0.9	3.1	Primary anemia. Hemoglobin 27 per cent. Red blood count 1.7 millions.
" O.	12	12	88	88	44	48	48	48	42.2	42.2	1.8	5.8	0.0	4.0	Primary anemia. Hemoglobin 27 per cent. Red blood count 0.9 million.
Anemia average.	12.5	12.5	87.5	87.5	51.0	55.0	56.2	57.0	**49.2**	**49.8**	**1.8**	**5.2**	**0.6**	**3.3**	
Mr. Cy.	45	45	55	55	66	72	98	100	53.9	55.0	12.1	17.0	1.1	4.9	Lymphatic leucemia. Hemoglobin 70 per cent. White blood count 39,000.
" Cd.	57	58	43	42	70	78	90	92	38.7	38.6	31.3	39.4	−0.1	8.1	Polycythemia vera. Hemoglobin 195 per cent. Red blood count 8.2 millions.
" F.	43	44	57	56	50	58	60	60	34.2	33.6	15.8	24.4	−0.6	8.6	Lobar pneumonia.
" P.	44	44	56	56	48	58	58	60	32.4	33.6	15.6	24.4	1.2	8.8	Cardiorenal disease.
Miscellaneous average.	47.2	47.7	52.7	52.2	58.5	66.5	76.5	78.0	**39.8**	**40.2**	**18.7**	**26.3**	**0.4**	**7.6**	

* A = arterial; V = venous.

the plasma while 7.6 volumes per cent are gained by the cells. The minimum and maximum figures on this same series show that the plasma gains as it becomes venous from 0.0 to 1.8 volumes per 100 volumes of whole blood, while the cells gain from 4.2 to 10.4 volumes. The obvious conclusion from these figures is that the transport of CO_2 is accomplished mainly by the cells.

Findings in Disease.

When we turn to the findings in anemia we meet with much the same thing. In a series of fourteen venous bloods from as many patients with anemia (Table I) the average CO_2 content of the whole blood was 58 volumes per cent which is essentially the same as that of the normal series. As one might suppose, of this 58 volumes per cent relatively more is in the plasma, 53 volumes per cent, and less in the cells, 5 volumes per cent, than in the case of the normal subjects. This altered distribution appears to be merely the result of the reduction in the number of cells in anemia.

When we come to compare arterial and venous samples in anemia (Table II), we find that here as in health it is the cells that are chiefly concerned in CO_2 transport. When we calculate the quantity of CO_2 carried in the plasma of arterial blood we get an average figure of 49.2 and for venous blood 49.8 volumes per cent. The cells of arterial blood, however, contain an average of 1.8 and those of venous blood 5.2 volumes per cent. As the blood passes from artery to vein, therefore, it gains an average of 4 volumes per cent, but of this only 0.6 volume per cent is gained by the plasma, while 3.3 volumes per cent, or more than five times as much, are gained by the cells. In anemia then, as in health, the conclusion may be drawn that the cells are more important in CO_2 transport than is the plasma.

In Table II observations on four patients with other diseases are shown. In Mr. F. with lobar pneumonia, and Mr. P. with cardiorenal disease, all factors and relationships are essentially like those of the normal series. Mr. Cy. with leucemia, and Mr. Cd. with polycythemia vera, on the other hand, both present a marked increase in the CO_2 content of their whole blood. A study of the distribution shows that in the former the amount in the cells is normal but the amount in the plasma increased, while

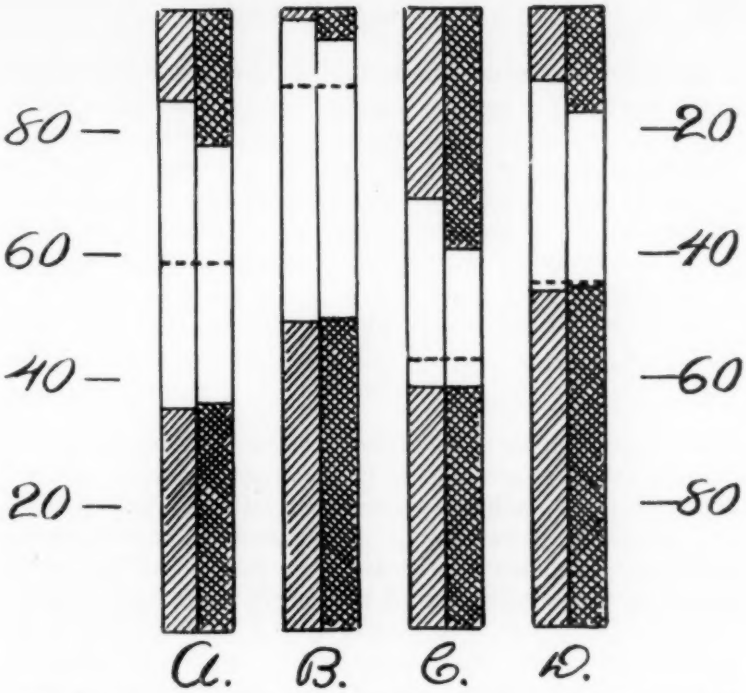

Fig. 1. Showing the distribution of CO_2 between cells and plasma in health and disease.

The entire column represents 100 cc. of blood. The portion above the horizontal interrupted line represents cells, that below, plasma, in accordance with the hematocrit findings. The upper shaded area shows the volume of CO_2 actually contained in the cells, and the lower shaded area that contained in the plasma of the given blood. Scales are provided so that these volumes may be read either from the top or bottom. The lightly shaded portions are for arterial, the heavily shaded, for venous blood.

Column A shows the relationships found in an average of eight normal bloods; Column B those of an average of four anemia bloods; Column C shows those of a patient with polycythemia vera; and Column D those of a patient with lymphatic leucemia.

It will be noted that in all four sets of columns the gain in CO_2 as blood passes from the arterial to the venous phase, is confined largely to the cells.

The difference in distribution of CO_2 between cells and plasma seems except in Column D to be dependent upon the relative amounts of cells and plasma in the several conditions. Anemic patients with a small proportion of cells carry less CO_2 in cells than normal persons and conversely the polycythemic patient carries more. In the leucemic patient (Column D), there is an actually greater quantity of CO_2 in the plasma which is not explainable on the ratio of cells to plasma.

in the latter the increase is in the load of the cells, the plasma load being normal. The increased amount in the cells in polycythemia is the converse of the condition in anemia and is therefore quite understandable. The cause for the increased amount of CO_2 in the plasma in the leucemia patient is less obvious. In both these patients in spite of the alteration in the distribution, the transport of CO_2 as in the other conditions studied is effected chiefly by the cells.

These relationships are shown graphically in Fig. 1.

DISCUSSION.

We do not propose to enter into an elaborate discussion of why the cells take a greater part in CO_2 transport than does plasma, nor of the actual method of its transport within the cells, whether as inorganic bicarbonate or in actual combination with hemoglobin as hemoglobin bicarbonate. Our purpose at the present time is merely to record certain phenomena as we found them.

It will not be out of place, however, to call attention to the greater and greater importance that, in the recent literature (1–3, 9), is being assigned to the rôle of hemoglobin in the matter of neutrality regulation and gas transport. It seems to be reasonably well established that hemoglobin is as important in the transport of CO_2 as it is in that of oxygen. Furthermore the recent work of Haggard and Henderson (10) seems to show that the corpuscles also regulate the bicarbonate reserve of the plasma.

Our findings, then, that both in health and disease the cells are chiefly concerned in CO_2 transport are not surprising. Our studies were on the actual relationships as they exist *in vivo*, but our results nevertheless seem to be essentially in agreement with those obtained with defibrinated blood *in vitro* by Joffe and Poulton (9). These writers found that the effect of oxygenation is to cause a loss of CO_2 which is more than twice as great for the corpuscles as for the plasma, and they draw the same conclusion, as do we, that "the transport of CO_2 in the blood is chiefly carried out by the corpuscles."

CONCLUSIONS.

1. As the blood passes from the arterial to the venous side of the circulation in normal man its cells gain from 4 to 11 volumes per cent of CO_2. At the same time the corresponding gain in the plasma is only from 0.0 to 1.8 volumes per cent. The conclusion is drawn, therefore, that the transport of CO_2 is accomplished mainly by the cells.

2. The same holds true in anemia and in certain other diseases, even though because of alterations in the cell volume: plasma volume ratio the actual distribution of CO_2 between cells and plasma is altered.

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RELATIONSHIP BETWEEN CHOLESTEROL AND CHOLESTEROL ESTERS IN THE BLOOD DURING THEIR ABSORPTION.*

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In a previous paper (1) it was shown that during the absorption of a neutral fat (olive oil) there is a marked increase in the blood of the cholesterol esters but no increase in the total cholesterol. Following the same experimental technique it was thought that it would be advisable to determine the relationship between the total cholesterol and the cholesterol esters in the blood during the absorption of free cholesterol and of cholesterol esters respectively.

That cholesterol is absorbed from the food and that the absorption is followed by a rise in the blood cholesterol is considered as established.

The first work of any significance was reported by Pribram (2) in 1906 who found that by the feeding of pure cholesterol and cholesterol esters respectively an increase in the blood of rabbits resulted. The increase was in part at least due to free cholesterol, as the serum from such animals showed an increased inhibitory power toward the hemolytic action of saponin, the free cholesterol alone being active as an inhibitory agent. Kusumoto (3) also showed indirectly that cholesterol must be absorbed as he found that 30 per cent of the amount ingested failed of excretion through the intestine. Gardner and his coworkers (4) in extensive studies on the origin and destiny of cholesterol in the animal organism came to the conclusion that cholesterol and cholesterol esters are absorbed into the blood, that the cholesterol esters are hydrolyzed before absorption, and that there results an increase in both free cholesterol and cholesterol esters in the blood after feeding cholesterol. Lehman (5) found that feeding cholesterol to rabbits results in an increase of the cholesterol in the blood in a few hours. In a series of experiments on dogs with a fistula in

* This investigation was supported in part by a grant from the Elizabeth Thompson Science Fund.

the thoracic duct Mueller (6) found that cholesterol was readily absorbed in the chyle. When cholesterol was fed either in the free form or in the form of esters there resulted an increase in both of these in the chyle, the proportion between the two remaining approximately the same as normally. These results indicated that either in the intestine or in the walls of the intestine processes of esterifications or hydrolysis take place depending upon the character of the material fed.

In the present investigation dogs were given a diet containing pure cholesterol or esters of cholesterol and blood specimens taken at once and at 2 hour intervals for 8 hours. Previous to the experiment the dogs were fasted for 24 hours. The blood specimens were drawn from the jugular vein into a hypodermic syringe (containing a little sodium citrate) and run at once into a centrifuge tube containing a little citrate. 3 cc. of the whole blood were then extracted and made up to 100 cc. volume according to Bloor's procedure (7). Then the tube was centrifuged for 30 minutes at 2,000 revolutions per minute and relative volume of corpuscles and plasma noted. 3 cc. of the plasma were then extracted as was the whole blood.

Total cholesterol and cholesterol esters were determined on each specimen of whole blood and plasma. Cholesterol was determined by Bloor's method (8) and cholesterol esters were determined by Bloor and Knudson's method (9).

Nine experiments were carried out, five of which were on diets containing free cholesterol and four on diets containing esters of cholesterol. In all the experiments, fat was excluded from the diet. A brief description of the experiments follows.

Experiment 1.—Dog 4 was a healthy male weighing 11.3 kg. It was fed a diet consisting of 4 gm. of cholesterol, 30 gm. of cracker meal, and 100 cc. of water at 9.10 a.m. At 9.15 a.m. the first specimen of blood was taken. After taking a 3 cc. sample for whole blood, the remainder was centrifuged and corpuscles were found to be 50.5 per cent. The plasma was clear and a 3 cc. sample was taken for analysis. The other specimens were as follows.

Specimen No.	Time.	Corpuscle.	Plasma.
		per cent	
2	11.20 a.m.	47.2	Clear.
3	1.20 p.m.	46.2	"
4	3.22 "	45.0	"
5	5.20 "	45.5	"

Experiment 2.—Dog 4 as above, weight 11.3 kg. Fed a diet containing 4 gm. of cholesterol, 30 gm. of cracker meal, and 100 cc. of water at 8.40 a.m. Specimens as follows.

Specimen No.	Time.	Corpuscle.	Plasma.
		per cent	
1	8.47 a.m.	48.5	Clear.
2	10.42 "	48.0	Slight turbidity.
3	12.42 p.m.	45.3	" "
4	2.50 "	45.0	" "
5	4.45 "	45.0	" "

Experiment 3.—Dog 6 was a healthy male weighing 10.4 kg. Fed a diet containing 3 gm. of cholesterol, 25 gm. of bread, and 150 cc. of water at 8.50 a.m. Specimens as follows.

Specimen No.	Time.	Corpuscle.	Plasma.
		per cent	
1	8.50 a.m.	38.0	Clear.
2	10.50 "	35.8	"
3	12.50 p.m.	36.0	"
4	3.00 "	37.0	"
5	4.50 "	36.8	"

Experiment 4.—Dog 8 was a healthy female weighing 8.5 kg. Fed a diet containing 3 gm. of cholesterol, 100 gm. of lean chopped beef, and 150 cc. of water at 12 m. Specimens as follows.

Specimen No.	Time.	Corpuscle.	Plasma.
		per cent	
1	12.10 p.m.	41.8	Clear.
2	2.10 "	42.0	"
3	4.10 "	41.9	"
4	6.05 "	41.5	"
5	8.05 "	42.1	"

Experiment 5.—Dog 8 as above, weight 9 kg. Fed a diet containing 2.5 gm. of cholesterol, 100 gm. of lean chopped beef, and 200 cc. of water at 11.25 a.m. Specimens as follows.

Specimen No.	Time.	Corpuscle.	Plasma.
		per cent	
1	11.30 a.m.	40.5	Clear.
2	1.35 p.m.	42.5	Slight turbidity.
3	3.30 "	40.4	Turbid.
4	5.30 "	42.66	Slight turbidity.
5	7.30 "	42.9	Turbid.

Experiment 6.—Dog 4 as above, weight 12.5 kg. Fed a diet containing 2.5 gm. of cholesterol palmitate, 50 gm. of bread, and 150 cc. of water at 8.40 a.m. Specimens as follows.

Specimen No.	Time.	Corpuscle.	Plasma.
		per cent	
1	8.45 a.m.	39.0	Clear.
2	10.50 "	37.0	"
3	12.55 p.m.	35.1	"
4	2.55 "	37.0	"
5	4.55 "	36.0	"

Experiment 7.—Dog 6 as above, weight 10 kg. Fed a diet containing 2.5 gm. of cholesterol oleate, 25 gm. of lean chopped beef, and 100 cc. of water at 8.50 a.m. Specimens as follows.

Specimen No.	Time.	Corpuscle.	Plasma.
		per cent	
1	9.00 a.m.	39.5	Clear.
2	10.50 "	36.0	Slight turbidity.
3	12.50 p.m.	36.2	Clear.
4	2.50 "	37.3	Slight turbidity.
5	4.50 "	36.6	" "

Experiment 8.—Dog 8 as above, weight 9 kg. Fed a diet containing 2.5 gm. of cholesterol stearate, 100 gm. of lean chopped beef, and 200 cc. of water at 11.20 a.m. Specimens as follows.

Specimen No.	Time.	Corpuscle.	Plasma.
		per cent	
1	11.30 a.m.	42.6	Clear.
2	1.35 p.m.	40.6	Slight turbidity.
3	3.50 "	42.0	" "
4	5.30 "	42.3	Turbid.
5	7.30 "	42.0	Slight turbidity.

Experiment 9.—Dog 11 was a healthy female weighing 20.5 kg. Fed a diet containing 2.5 gm. of cholesterol palmitate, 25 gm. of sugar in 200 cc. of a 1 per cent gum tragacanth solution at 9.55 a.m. Specimens as follows.

Specimen No.	Time.	Corpuscle.	Plasma.
		per cent	
1	10.05 a.m.	57.8	Clear.
2	12.00 m.	57.4	"
3	2.05 p.m.	57.0	Slight turbidity.
4	4.00 "	57.3	Clear.
5	6.00 "	57.8	"

TABLE I.

Cholesterol and Cholesterol Esters During Their Absorption per 100 Cc.

No. of experiment.	Time.	Total cholesterol.			Cholesterol as esters.		
		Whole blood.	Plasma.	Corpuscles.	Whole blood.	Plasma.	Corpuscles.
	hrs.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
1	Before.	211	215	208	73	148	1
	2	253	195	318	72	149	4
	4	301	298	326	72	136	2
	6	306	309	302	71	135	6
	8	324	324	324	70	134	6
2	Before.	225	225	225	71	136	−2
	2	250	258	241	73	138	−2.6
	4	268	277	256	74	130	+5
	6	258	277	236	73	134	−1
	8	294	309	277	70	132	−5
3	Before.	170	168	173	70	111	+2.6
	2	190	192	187	69	107	0
	4	201	203	198	74	113	+4.2
	6	208	211	202	70	108	−5.4
	8	193	178	217	71	115	−4
4	Before.	193	185	204	70	125	−6
	2	219	215	225	71	124	−2.3
	4	238	220	264	70	126	−7
	6	208	192	231	69	123	−7
	8	263	250	280	72	131	+4.7
5	Before.	190	193	186	81	136	0
	2	205	200	211	80	140	−1
	4	223	206	247	78	134	−4.9
	6	245	213	287	78	132	+4.6
	8	219	200	245	80	140	0
6	Before.	167	160	178	78	125	+4
	2	192	174	223	80	125	+2.7
	4	203	178	251	78	120	0
	6	196	195	200	79	122	+5.4
	8	177	168	194	79	121	+4.1

TABLE I—*Concluded.*

No. of experiment.	Time.	Total cholesterol.			Cholesterol as esters.		
		Whole blood.	Plasma.	Corpuscles.	Whole blood.	Plasma.	Corpuscles.
	hrs.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
7	Before.	172	177	165	71	117	0
	2	179	185	170	72	114	−2.7
	4	217	214	221	75	116	+1.9
	6	205	208	201	73	115	+2.6
	8	183	194	164	70	111	−1.2
8	Before.	193	192	194	77	136	−2.4
	2	211	208	215	77	132	−3.7
	4	231	227	237	80	138	0
	6	250	238	262	79	132	+5.9
	8	250	234	271	83	140	+4.5
9	Before.	185	181	188	62	145	+1.3
	2	209	208	213	64	152	−1
	4	221	221	221	66	147	+4.7
	6	260	268	255	66	147	+5.2
	8	236	238	235	60	140	+1.7

The dogs used in these experiments were previously accustomed to handling, and did not struggle during the experiments or show other evidences of excitement, so those factors do not influence these results.

The analytical results are given in Table I. Direct determinations were made only on the whole blood and plasma from which, since the percentage of corpuscles in the blood was known, the composition of the corpuscles was calculated.

DISCUSSION.

The total cholesterol is found to be increased in all the experiments both in feeding cholesterol and cholesterol esters. This result is in accord with the work of other investigators (2–6). The increase in the whole blood varies from 21.5 per cent in Experiment 6 to 53.5 per cent in Experiment 1 and averages 32.3 per cent. The greatest increase occurs in most of the experiments either in the 6th or the 8th hour after feeding. Comparing

the increase of cholesterol in the plasma and corpuscles it is observed that the increase in the corpuscles is greater in seven of the nine experiments.

The values for cholesterol esters show that the cholesterol in combined form remains the same throughout the periods of observation. Even though the total cholesterol increases as much as 50 per cent in the whole blood the amount of cholesterol as esters does not show any appreciable change. The values for cholesterol esters in the corpuscles indicate that the corpuscles probably contain no combined cholesterol which bears out the assumption generally made. Of course it must be remembered that the values for cholesterol esters in the corpuscles are calculated from the amount of them in the whole blood and plasma and as a consequence are not so reliable as when determined directly. This will also explain why some values in the table are given in negative quantities.

The results of these experiments indicate then that when cholesterol or cholesterol esters such as palmitate, oleate, or stearate are fed in a diet there results a marked increase in the total cholesterol in the blood but no change in the amount of cholesterol as esters. The cholesterol esters must as a consequence be hydrolyzed before absorption in the intestine and apparently are not synthesized again before passing into the blood.

These results do not agree with the observations reported by Gardner and his coworkers (4) who found that when cholesterol was fed to rabbits there resulted an increase in the cholesterol esters as well as cholesterol.

The experiments of Gardner were carried out by feeding a diet to which cholesterol was added and after several days killing the animal and analyzing the blood. In these experiments it may be possible that the level for cholesterol in the tissues and blood was raised after feeding it for several days and as a result an equilibrium is established between free cholesterol and cholesterol esters in the blood and tissue resulting in an increase of cholesterol esters as well as free cholesterol. Recently Hueck and Wacker (10) have reported that cholesterol fed to rabbits causes an increase in the esters in the blood. Their experiments were similar to those of Gardner and his coworkers but were carried over a longer period. They fed rabbits for a period of 181 days in one experiment and 126 days in another and in that time the rabbits had received 220 and 130 gm. of chol-

esterol respectively. Due to the large amount of cholesterol fed it would be expected that there would be an accumulation of cholesterol in the tissues and blood and the relation between the free and bound cholesterol would tend in time to be constant. In neither of these investigations was the blood examined just before feeding of cholesterol diets and at short intervals during the period of absorption so that the results are not comparable with mine.

However, Mueller (6) found that there resulted an increase in both cholesterol and cholesterol esters in the chyle of dogs when either one was added to the diet. Mueller's results might be partially explained by the fact that in the diets containing cholesterol or cholesterol esters fat was also present. I have shown in a previous paper (1) that during absorption of fat alone there results a marked increase of the cholesterol esters in the blood so that the fatty acids resulting from the hydrolysis of the fat in Mueller's experiments are probably transported in part combined with the cholesterol. In all my experiments I excluded fat from the diets except very small amounts present in those experiments where I used some lean beef, bread, or cracker meal. By omitting the fat there was no excessive amount of fatty acid to be transported by the blood.

SUMMARY.

A series of experiments carried out on dogs feeding them cholesterol and cholesterol esters, respectively, shows that free cholesterol is increased considerably in all the experiments during the absorption of the diets but that there is no apparent change in the amount of cholesterol in the ester form.

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PENTOSE MONONUCLEOTIDES OF THE PANCREAS OF THE DOGFISH (SQUALUS SUCKLII).

PRELIMINARY COMMUNICATION.

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Two mononucleotides in which a pentose, *d*-ribose, unites purine and phosphoric acid are known to occur in the animal body; guanylic acid and inosinic acid. The former contains guanine as purine base, the latter hypoxanthine. The corresponding compound with adenine has been shown to be one of the component mononucleotides of yeast nucleic acid by Jones and Kennedy (1) and by Levene (2); but it does not appear to have been found in the animal body. Guanylic acid, originally isolated by Hammarsten (3) from the β-nucleoprotein of the pancreas of the ox, has since been found widely distributed in mammalian glands (4, 5). Inosinic acid, first isolated by Liebig (6) from meat extract, is now known to be a constituent of the muscle of many mammals and of some birds and fishes (7). It has not, however, been identified as a gland constituent in any animal and the wide distribution of pentoses in animal tissues has generally been attributed to the presence of guanylic acid (8).

It is the object of the present communication to record evidence pointing toward the occurrence of adenosinphosphoric acid and inosinic acid, in addition to a guanylic acid, in the pancreas of the dogfish.

The essentials of Hammarsten's method of preparation of β-nucleoprotein from ox pancreas are rapid extraction of the material with boiling water and precipitation from the cooled extract by dilute acid. The crude material may be purified by dissolving in dilute alkali and reprecipitating with dilute acid repeatedly and yields an alkali albuminate and guanylic acid on heating at water bath temperature with 2 per cent potash

(9). According to Bang the acid is precipitated on neutralizing the alkaline digest and cooling. He points out, however, that it is soluble in water to the extent of about 0.3 per cent, but only sparsely soluble in dilute acetic acid. Levene and Mandel (5), working with liver nucleoprotein, obtained precipitation by concentrating the neutral solution strongly at low temperature, while Jones and Rowntree (4), utilizing material obtained from pigs' spleen, find it necessary to add acetic acid to cause the guanylic acid to separate.

Inosinic acid occurs either free or in readily broken down combination in the muscle tissues in which it has been found. There is no evidence that it forms any protein combination. It is readily extracted in water and cannot be precipitated from the extract either by boiling or acidification. Several methods have

TABLE I.

Experiment No.	Pentose (as xylose).		Total pentose (xylose) remaining in acetic acid filtrate.
	Extract.	Acetic acid filtrate.	
	per cent	*per cent*	*per cent*
1	0.0226	0.0114	50.4
2	0.0358	0.0217	60.6

been described for its isolation which, together with a new one, are summarized by Bennett (7). All, except the original method of Liebig, depend upon precipitation of the acid either as the lead or the silver salt. In a recent communication (10) attention was drawn by the writer to the occurrence of a substance, or substances, in boiling water extracts of various tissues of marine animals which yielded furfurol in relatively large quantity on heating with 12 per cent hydrochloric acid, and probably therefore contains a pentose, and was not precipitated on adding acetic acid to the extract.

The fraction of the furfurol-yielding substances in the extract which remained in solution after complete precipitation with acetic acid was by no means insignificant. The figures in Table I were, for instance, obtained from extracts of dogfish pancreas and the corresponding acetic acid filtrates.

Grund's modification of the method of Tollens and Kröbe for the determination of pentoses (11) was used and calculation made to xylose. It was necessary to concentrate the acetic acid filtrates in order to obtain a sufficiently large yield of furfurol from the prescribed volume of liquid. The samples taken for analysis were of such a volume that, after making up to 100 cc. with concentrated hydrochloric acid, the solutions contained the necessary 12 per cent of acid.

Interest was first aroused in the presence of these acetic acid-soluble furfurol-yielding compounds from the point of view of its possibly indicating pentosans in the tissues of the animals examined. Pentosans are largely represented in the algæ which, directly or indirectly, make up their diet; moreover it has been stated that such compounds occur in certain mollusks living on algæ (12). It was found, however, that in all the cases examined the pentose compounds could be completely precipitated from the acetic acid solution by alcohol and that the precipitates thus obtained invariably contained both phosphoric acid and purine in conjugation, so that it seemed more probable that the pentose was present in nucleotide combination.

The tissue selected for examination with a view to the more precise determination of the nature of the combination was the pancreas of the dogfish for two reasons; first, because it was the tissue richest in pentose of all those examined and, second, because the pancreas is the classical seat of guanylic acid in mammals and it seemed of special interest to determine whether the same compound occurs in the lower vertebrates of which the dogfish may be taken as a type.

EXPERIMENTAL.

The property of yielding furfurol, which is easily recognized by its reaction with aniline acetate, phloroglucin, orcinol, etc., on heating with 12 per cent hydrochloric acid, has been taken as evidence of the presence of combined pentose in the preparations under examination. The only other class of compounds which might have been present and which yield furfurol under the same conditions is the conjugated glucuronic acids. Practically all these compounds are, however, soluble in alcohol and ether (13) and the material used in this work was found to give up none

of its furfurol-yielding constituents by exhaustive extraction with both solvents. Starkenstein and Henze (12) consider that glucosamine, which occurs in the tissues combined as glucoprotein, is responsible for the furfurol reactions with orcinol and phloroglucin given by preparations from the livers of certain mollusks. It was found, however, that glucosamine chloride, prepared from crab shells, did not yield a trace of furfurol on heating with 12 per cent hydrochloric acid and concluded, therefore, that compounds of this class might reasonably be excluded from consideration. Bial's orcinol reagent was frequently used to detect pentose, but in every case where sufficient material was at hand to make comparisons possible it was found that a positive reaction with this reagent indicated a positive one on distilling with 12 per cent hydrochloric acid, in accordance with the Tollens method for pentose determination, and testing the distillate with aniline acetate or phloroglucin.

The test for purines has been carried out by the Krüger-Schmid procedure (14) followed by production of the silver compounds by precipitation with ammoniacal silver nitrate from the solution obtained by hydrogen sulfide decomposition of the copper salts. Purines were freed from combination by boiling with 5 per cent sulfuric acid. Free phosphoric acid was detected by magnesia mixture and the conjugated acid by ammonium molybdate after boiling with nitric acid.

The pancreas was excised from forty-nine freshly killed dogfish and freed as far as possible from adherent mesentery. The material weighed 495 gm. It was put through a mincer and immediately put into rapidly boiling water, boiled for 5 minutes and strained through close cloth, squeezed out, and extracted in the same way a second time. The residual material after this treatment gave only a trace of furfurol on distillation with 12 per cent hydrochloric acid. The united extracts measured 2.25 liters. After cooling, the liquid was decanted from solid material which had settled out and, after repeated filtration, was obtained in an only slightly turbid condition. 20 cc. of glacial acetic acid were added which produced a heavy precipitate. This was allowed to settle and left a perfectly clear fluid which was partly decanted and partly filtered off.

The precipitate was washed repeatedly by decantation with 1 per cent acetic acid and finally put upon a filter. It gave a strong pentose reaction and contained conjugated phosphoric acid, purine, and protein. An attempt was made to purify the material by dissolving it in cold 1 per cent sodium hydroxide and reprecipitating with acetic acid. It was found that the pentose-containing substance could not be completely reprecipitated by dilute acid or by adding a considerable excess and that, after repeating the operation twice, only protein and a trace of phosphoric acid could be detected in the precipitate, but no purine or pentose. The nucleoprotein was evidently split by treatment with alkali and the non-protein constituent remained in solution in acetic acid.

The acetic acid filtrates were accordingly neutralized and concentrated to small volume. The solution contained no protein and no free pentose, purine, or phosphoric acid. All were present in the conjugated condition. The solution was poured into a large volume of alcohol which produced a voluminous precipitate giving all the reactions of the original material and containing all the combined pentose. The precipitate was dissolved in 100 cc. of 5 per cent sulfuric acid and heated at water bath temperature for 2½ hours. After cooling, the solution was neutralized and the purines were precipitated as cuprous compounds by the Krüger-Schmid procedure (14). The precipitate was suspended in hot water, decomposed with hydrogen sulfide, and, after boiling off excess hydrogen sulfide, the liquid with copper sulfide in suspension was boiled with sulfuric acid and filtered. The filtrate was neutralized with ammonia, 2 per cent excess added, and warmed. A precipitate separated immediately. After digesting on the water bath for 1 hour and standing over night this was filtered off, washed with 1 per cent ammonia and water, and purified by dissolving in 1 per cent sodium hydroxide and reprecipitating with acetic acid. The material so obtained was further identified as guanine by the crystalline form of its hydrochloride and the melting point of the picrate prepared from it (159–160°C.).

The ammoniacal filtrate from the guanine precipitation was treated with silver nitrate which produced a heavy precipitate. This was filtered off, washed, and decomposed with a small quantity of warm hydrochloric acid. The filtrate from silver

chloride gave no trace of precipitate on making 2 per cent ammoniacal and warming. Guanine was therefore absent. The solution was boiled till free from ammonia, made just acid with hydrochloric acid, and excess of a saturated solution of picric acid added. A characteristic light yellow precipitate of adenine picrate immediately separated which was filtered off, washed, recrystallized from boiling water, and further characterized as adenine picrate by its crystalline form and melting point (180–181°C.).

The filtrate from adenine picrate was freed from picric acid by means of sulfuric acid and ether, made ammoniacal, and tested with silver nitrate. Absence of precipitate indicated that the solution contained no further purines.

The precipitate produced by acetic acid from the boiling water extract of dogfish pancreas contains therefore, in addition to protein, pentose, and conjugated phosphoric acid, guanine and adenine as purine bases.

The acetic acid filtrate from this precipitate was neutralized and evaporated to 470 cc. at 60–70°C. It was cooled at this stage and filtered from a small amount of coagulated material. It contained conjugated purine, pentose, and phosphoric acid, but only a trace of phosphoric acid in the free condition. 1 per cent of acetic acid was added and the liquid allowed to stand over night. No precipitate was formed. It was further concentrated to 100 cc. and again treated with acetic acid, but still without result even after prolonged standing. Guanylic acid similar to that isolated from mammalian material does not, therefore, appear to be present. The solution was poured into a large volume of alcohol which produced a heavy gelatinous precipitate which gave a slight biuret reaction, but no xanthoproteic, and did not react with Millon's reagent. It contained conjugated phosphoric acid, purine, and all the combined pentose present in the original solution, but none of the compounds in a free condition.

In order to break down any protein combination present the material was dissolved in 100 cc. of 2 per cent caustic potash and heated on the water bath for ½ hour. The solution thus obtained was cooled, acidified with acetic acid, and allowed to stand for 18 hours. No precipitation took place. The solution was neu-

tralized and treated with saturated barium hydroxide until precipitation was complete to remove any free phosphoric acid or protein decomposition products present. The precipitate was filtered off and washed. The filtrate gave no biuret reaction. It was neutralized with acetic acid and treated with basic lead acetate solution until precipitation was just complete. The lead precipitate was thoroughly washed, suspended in water, and decomposed with hydrogen sulfide, excess hydrogen sulfide removed by an air current, and excess barium carbonate added. After boiling, the lead sulfide and barium carbonate were filtered off and the filtrate was evaporated to a small volume at as low a temperature as possible on a water bath. It was then poured into five volumes of alcohol which produced a bulky gelatinous precipitate containing all the pentose. This was filtered off with suction, dried at room temperature, and dissolved in hot water. On cooling, the solution deposited a white powdery substance which increased on standing over sulfuric acid in an exhausted desiccator for some days, but no definite crystalline form could be made out even after recrystallizing. After slow evaporation of the solution nearly to dryness this material was filtered off, washed with alcohol and ether, and dried. It gave positive tests for pentose, purine, conjugated phosphoric acid, and barium. It was redissolved in water, the barium removed with sulfuric acid, and the acid strength made up to 5 per cent. After heating the solution on a water bath for 3 hours it was neutralized and the same procedure followed for the separation of the purines as in the case of the acetic acid precipitate from the original extract. No guanine could be detected and only a small quantity of adenine, but the filtrate from adenine picrate gave a precipitate with ammoniacal silver nitrate indicating the presence of further purine compounds. The silver nitrate precipitate was accordingly decomposed with hydrochloric acid and the solution of chlorides thus obtained evaporated cautiously to dryness, moistened, and evaporated a second time. The residue gave no xanthine reaction with nitric acid and soda and was completely soluble in warm water. Xanthine was therefore not present. The copper precipitation was repeated and the residue obtained by evaporation of the solution resulting from hydrogen sulfide decomposition of the precipitate crystallized from 6 per cent nitric acid. Char-

acteristic whetstone-shaped crystals of hypoxanthine nitrate were thus obtained.

The filtrate from the acetic acid precipitation of the boiling water extract of the dogfish pancreas is thus shown to contain hypoxanthine and adenine as purine bases as well as conjugated phosphoric acid and pentose.

It seemed possible to interpret the results thus far obtained on the assumption that only ordinary α-nucleoprotein (the compound of protein with nucleic acid of the thymus nucleic acid type) and ordinary β-nucleoprotein (the compound with guanylic acid) were in question and that:

1. α-nucleoprotein had been extracted from the pancreas together with β-nucleoprotein and the adenine in the acetic acid precipitate was due to nucleic acid, the pentose being combined only with guanine.

2. Nucleic acid had been broken down by enzyme action leading to the production of adenine hexose nucleotide or nucleoside and a corresponding hypoxanthine compound, and both of these had remained in solution after treatment of the extract with acetic acid. Since, however, both combined guanine and free pentose are absent from the acetic acid solution the combined pentose present would in this case have to be attributed to the pentose-phosphoric acid compound split off from guanylic acid.

The first possibility seemed unlikely because the essence of the method of separation of α- and β-nucleoproteins is the insolubility of the former in boiling water;[1] the second because of the expeditiousness with which the tissues had been brought into boiling water. Moreover, the assumption made in this latter case to account for the presence of pentose necessitates a splitting off of free guanine from guanylic acid. However, no free purine was found either in the original acetic acid precipitate or in the filtrate from that precipitate, and there seemed no reason to anticipate that either boiling with water or the action of cold dilute acetic acid could have caused such a split.

[1] The only difference in the procedure used here and that usually described for extracting β-nucleoprotein from tissues is that the material has been placed directly in boiling water, in order to eliminate risk of enzyme action during extraction, instead of putting it into cold water and raising to the boiling point.

Jones (15) has shown in the case of the pig that the splitting off of guanine from guanylic acid is not brought about under normal conditions by pancreatic enzymes. Thus both purely chemical and (as far as it is justifiable to argue from the pig to the dogfish) biochemical considerations indicate that the presence of combined pentose in the acetic acid filtrate of the extract of dogfish pancreas cannot be attributed to breakdown of guanylic acid with the elimination of guanine.

The entire absence of xanthine from both the precipitates and filtrates obtained from the extract also argues against any enzyme action having intervened in the course of the operations if analogy is justifiable between ferment conditions in the pancreas of the dogfish and that of the pig, since, in the latter case, xanthine is invariably a product of autolysis (15).

There seems, therefore, no alternative but that the pentose in the acetic acid filtrate is combined with preformed adenine or hypoxanthine, or both.

It seemed probable that if more material were taken at the outset it might be possible to separate the compounds of guanine, adenine, and hypoxanthine more completely and thus determine with greater certainty with which of the purines the pentose was combined.

To this end the pancreas was excised from 140 dogfish and the material, which weighed 921 gm., ground, and extracted with boiling water as in the previous instance, and the extract cooled and filtered. About 5 liters of extract were obtained which were concentrated at a low temperature to 1,500 cc. During concentration a small amount of suspended material coagulated and was filtered off after cooling. The filtrate was only very slightly turbid. 30 cc. of glacial acetic acid were added and the precipitate produced was separated and washed as before. The precipitate behaved as in the previous instance on endeavoring to purify it by alternate treatments with alkali and acetic acid and the non-protein constituents were obtained in solution in acetic acid.

This solution was neutralized and a saturated solution of normal lead acetate added until precipitation was complete. The lead salt was converted into the barium salt in the manner previously described. The barium salt was crystalline and difficultly soluble

in water. It contained conjugated pentose, purine, and phosphoric acid. It was freed from barium, hydrolyzed with 5 per cent sulfuric acid, and the purines present were determined as before. Guanine and adenine were found in about equal quantities but no xanthine or hypoxanthine.

The filtrate from the normal lead acetate precipitate was treated with basic lead acetate and a further precipitate obtained. This was converted into the barium salt as before. It had the same appearance and gave the same reaction as that obtained from the normal lead acetate precipitate and contained the same purine bases; adenine was, however, present in larger quantity than guanine.

The filtrate from the basic lead acetate precipitate, after removal of lead, gave no pentose reaction and was not further examined.

The purine constituents of the acetic acid precipitate are thus shown to be guanine and adenine as in the experiment previously described.

The filtrate from the acetic acid precipitation of the extract was neutralized and a saturated solution of barium hydroxide added until precipitation was complete. The precipitate thus obtained contained the pentose nucleotide constituents. It was decomposed with sulfuric acid, hydrolyzed as in the previous instances, and the purines present were determined. Again adenine and guanine, but no xanthine or hypoxanthine, were found. The fact that barium hydroxide carries down compounds of the same constituents as occur in the acetic acid precipitate from the filtrate from that precipitation was overlooked in the experiment previously described.

The filtrate from barium hydroxide precipitation still gave a strong pentose reaction. After neutralizing with acetic acid it failed to give a precipitate with normal lead acetate, but gave a heavy one on treatment with the basic salt. The filtrate from this precipitation was free from pentose nucleotide constituents. The lead salts obtained were converted into the barium salts which were finally crystallized from hot water. It was found possible to separate the material in this way into two fractions having a marked difference in solubility in water, but both giving all the reactions for pentose nucleotides and barium. The more

soluble fraction was the larger one. After removal of barium and hydrolysis with 5 per cent sulfuric acid it gave no reaction for guanine, but a heavy precipitate of adenine picrate. The filtrate from the picrate precipitation, after removal of excess picric acid by sulfuric acid and ether, gave a slight precipitate with ammoniacal silver nitrate which, after washing and dissolving in boiling nitric acid of 1.1 specific gravity, deposited on cooling characteristic crystals of hypoxanthine silver nitrate. The compound was further identified by removing the silver and crystallizing out typical whetstone-shaped crystals of hypoxanthine nitrate which gave no trace of xanthine reaction with nitric acid and caustic soda. The filtrate from hypoxanthine silver nitrate deposited a trace of red-brown precipitate on being rendered ammoniacal which, after removal of silver and evaporation of the solution of nitrate obtained, deposited a very slight residue giving a characteristic xanthine reaction.

The less soluble fraction of the barium salts was treated in the same way as the more soluble one. It contained neither guanine nor adenine, but only hypoxanthine and a trace of xanthine as purine constituents.

The filtrate from barium hydroxide precipitation of the acetic acid filtrate from the original extract contains, therefore, both adenine and hypoxanthine and a trace of xanthine in addition to conjugated phosphoric acid and pentose.

The results obtained from this experiment thus confirm the conclusion reached from the former one. For the reasons advanced in connection with that experiment coupled with the fact that, in the case just described, adenine is found with only a trace of hypoxanthine in one fraction of the barium salts obtained from the acetic acid filtrate of the extract, and hypoxanthine with practically no other base in the other fraction, it appears further that both adenine and hypoxanthine are present in mononucleotide combination.

The occurrence of the adenine compound in the acetic acid precipitate of the extract in both experiments indicates that this compound is present in the tissue in nucleoprotein combination. There is, however, no evidence that inosinic acid is similarly combined since hypoxanthine has not been found in the acetic acid precipitate.

Levene (2) has shown that adenosinphosphoric acid is best identified as the free acid obtained through the ammonium salt. It is hoped to accumulate sufficient of the more soluble fraction of the barium salt prepared as described to make identification along these lines possible.

Inosinphosphoric acid (inosinic acid) is usually identified as the barium salt. This is, according to Bennett (7), readily soluble in warm water, differing in this respect from the fraction of barium salts obtained as above described containing hypoxanthine as purine constituent. The compound obtained may be the basic salt. Haiser (16) has described such a sparingly soluble compound derived from inosinic acid by the action of excess barium hydroxide. This point also can be elucidated only on the accumulation of more material.

CONCLUSIONS.

Evidence has been advanced indicating that:

1. The β-nucleoprotein of the pancreas of the dogfish contains both adenine and guanine in pentose mononucleotide combination.

2. This β-nucleoprotein is readily broken down to its constituent mononucleotides and protein by the action of cold 1 per cent sodium hydroxide.

3. The mononucleotides thus obtained are not precipitated from either neutral or acetic acid solution. The guanine mononucleotide differs in this respect from the guanylic acid previously isolated from mammalian material.

4. The acetic acid filtrate from β-nucleoprotein precipitation contains the mononucleotides of the β-nucleoprotein in solution together with inosinic acid.

Addendum.—Since the foregoing paper was written a communication has appeared by Hammarsten[2] which bears directly on the findings recorded and has drawn the writer's attention to earlier papers by Feulgen[3] which also have an important bearing on the subject. Both these authors have found adenine as well as guanine in β-nucleoprotein prepared from the pancreas of the ox by the Hammarsten method. Feulgen, by enzymic decomposition of the β-nucleoprotein has isolated a compound of guanylic

[2] Hammarsten, E., *J. Biol. Chem.*, 1920, xliii, 243.

[3] Feulgen, R., *Z. physiol. Chem.*, 1914, xci, 165; 1919, cvii, 147.

acid with nucleic acid and Hammarsten has isolated the same, or a very similar, body by direct extraction of the dried gland with cold dilute alkali and precipitation with acid. The latter author has also found about one-third as much adenine as guanine in β-nucleoprotein by acid hydrolysis followed by precipitation of the purines as silver salts and fractionation by the method of Steudel, and, since these bases are shown to be present in about the same ratio in the conjugated nucleic acid isolated by direct extraction of the pancreas, the implication appears to be made that β-nucleoprotein may be a body of similar constitution. Both authors regard the adenine found as part of the nucleic acid (tetranucleotide) component of the conjugated nucleic acid.

It is possible that the adenine found in the acetic acid precipitate (β-nucleoprotein) from the extract of the pancreas of the dogfish is due to a conjugated nucleic acid such as has been described by these authors. The presence of such a compound in the extract would not, however, affect the conclusion which has been drawn as to the pentose nucleotide combination of the adenine found in the filtrate after precipitation by acetic acid and by barium hydroxide.

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A CRITIQUE OF EXPERIMENTS WITH DIETS FREE FROM FAT-SOLUBLE VITAMINE.*

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Practically all recent investigators who have fed mixtures of purified food products to small animals have either assumed or concluded from their experiments that lard is devoid of fat-soluble vitamine.[1] In fact the importance of the latter factor was discovered because of the nutritive failure of rats that were being fed on diets in which lard furnished the sole fat therein.[2,3] The results reported by numerous investigators have, however, differed in respect to the effect of such diets on young rats. While some, like ourselves, have described their animals as usually growing at a nearly, or quite, normal rate for periods of 60 to 80 days before showing signs of a nutritive deficiency in the diet, others have secured little if any growth under apparently similar conditions. All, however, agreed that such diets are defective and that the failure to maintain body weight was followed sooner or later by symptoms of disease and death unless some source of the fat-soluble vitamine was supplied. It was surprising therefore to learn from the recent paper of Daniels and Loughlin[4] that rats could be made to grow, reproduce, and rear their young ". . . .

* The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, Washington, D. C.

[1] Report on the present state of knowledge concerning accessory food factors, *Medical Research Comm., National Health Insurance, Special Rep. Series, No. 38*, 1919, 16.

[2] McCollum, E. V., and Davis, M., *J. Biol. Chem.*, 1913, xv, 167.

[3] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1913, xv, 311.

[4] Daniels, A. L., and Loughlin, R., *J. Biol. Chem.*, 1920, xlii, 359.

on diets in which the only apparent source of the vitamine was the lard or cotton-seed oil." These workers concluded that " . . . . both these fats contain appreciable amounts of the fat-soluble growth stimulant. This, however, is demonstrable only when fairly large amounts are fed."

In view of these divergent results it has seemed to us worth while to subject this problem to further critical investigation so that we might learn, if possible, the reason for the existing disagreement. In our own earliest observations[3] upon rats which were fed diets consisting of casein, edestin, or ovalbumin 18, "protein-free milk" 28, starch 26 to 29, lard 25 to 28 per cent; or casein, edestin, or lactalbumin 18, "artificial protein-free milk" 29, starch 25 to 28, lard 25 to 28 per cent, the characteristic nutritive failure expressed by retardation of growth followed by decline in body weight was not recognized until the lapse of many weeks. To avoid the uncertainty of possible changes resulting from the process of preparation for the market in the case of the commercial lard fed in these early trials, the experiments were duplicated by using lard rendered at comparatively low temperatures in our own laboratory.[5] The outcome with this carefully prepared fat was no different. The characteristic nutritive failure occurred in about the usual time, and the animals were restored to health by replacing a part of the lard with butter fat. The diets in these cases of malnutrition included 28 per cent of lard, an amount equal to that supplied by Daniels and Loughlin in rations of otherwise similar character. The failures were not confined to the use of one protein; for rats living on diets supplying casein, edestin, ovalbumin, or lactalbumin (as already indicated) showed the usual period of seemingly normal growth followed by failure, frequently with manifestation of eye disease. These and similar experiences are doubtless responsible for the following conclusion reached by the Committee upon Accessory Food Factors under the Medical Research Committee of Great Britain:[1]

" . . . . for a short time the young animals are able to grow when the fat-soluble factor is deficient in their diet. The explanation of this is probably supplied by the assumption that the animal organism normally

[5] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1915, xx, 379.

contains reserve supplies of the factor 'A' in its own body. If this hypothesis is correct it is reasonable to assume that these reserves are mobilized for use when a deficiency occurs in the diet, but as soon as they are exhausted growth is immediately inhibited. During the period of temporary growth, throughout which it has been assumed that the reserve supplies are being utilized, the animals show a normal appearance and there does not appear to be any decline in their health, but when the reserves are exhausted and the deficiency becomes felt, not only do they cease to grow but they become highly susceptible to bacterial infection."

In our own experiments just referred to, the indispensable water-soluble vitamine was furnished in the form of "protein-free milk." It has been alleged that the initial delay in the manifestation of nutritive damage was due to fat-soluble vitamine carried by this product. Subsequently we replaced the protein-free milk by an artificial mixture of inorganic salts and starch (in place of milk sugar) and supplied the water-soluble vitamine by adding dried brewery yeast. The outcome with diets made up of casein or lactalbumin 18, or meat residue 19.6, salt mixture 4, starch 47 to 52.4, lard 24 to 31 per cent, plus 0.2 to 0.6 gm. of dried brewery yeast daily, was not essentially different; the failure did not appear notably earlier than in the original experiments.[6] Separate trials showed the yeast to be devoid of fat-soluble vitamine, even enormous quantities of it failing to protect the animals on lard diets against the effects of the deficiency in fat-soluble vitamine[7] (see Chart I).

As already stated, there are in the literature of the subject several records[8] of young animals, fed on foods supposedly free from fat-soluble vitamine, which failed to grow in the way that we have described, whereas the experience of others[9] coincides with our own published results in which the nutritive decline was delayed until some considerable growth had been made. This raises the question of the complete freedom from fat-soluble vitamine of the foods used in the rations employed in different

[6] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1920, xli, 549, Charts I and III.

[7] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxvii, 199.

[8] McCollum, E. V., Simmonds, N., and Pitz, W., *Am. J. Physiol.*, 1916, xli, 333. Halliburton, W. D., and Drummond, J. C., *J. Physiol.*, 1917, li, 235.

[9] Stephenson, M., and Clark, A. B., *Biochem. J.*, 1920, xiv, 502.

investigations and the methods employed for purifying their ingredients. Were there traces of fat-soluble vitamine remaining in the products employed, which will account for the initial recorded growth of young rats on diets of protein, carbohydrate, inorganic salts, and lard?

The problem of the removal of this vitamine from food products has not been satisfactorily solved. Many protocols describe

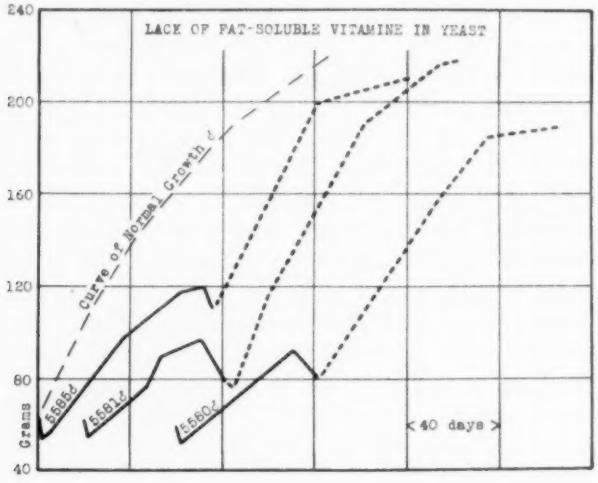

CHART I. Showing the failure of very large quantities of dried brewery yeast to furnish sufficient fat-soluble vitamine to protect rats from nutritive decline on diets otherwise adequate. Renewal of growth promptly ensued when butter fat was incorporated in the diet during the period indicated by the interrupted lines. The food mixture consisted of yeast 42.5, salt mixture 4, starch 30.5, lard 23 per cent.

the use of foods purified by extraction with various organic solvents, particularly ether. There is reason to believe that the fat-soluble vitamine is not always readily soluble in such liquids. Pure ether may be ineffective, in some cases at least, unless the food product has been previously treated with alcohol. This behavior is analogous to that of phosphatides in egg yolk, blood serum, milk, etc., which are insoluble in *pure* ether until they have been liberated from some combination with protein by

preliminary treatment with alcohol.[10] Although it has been reported[11] that ether does not extract the fat-soluble vitamine from dried leaves, we have obtained it by extraction with U. S. P. ether (which contains alcohol and water).[12] From the substances extracted by alcohol the vitamine is perhaps extractable by ether. The effect of heat on this food factor may hinge upon its preliminary liberation from other complexes.

Inasmuch as the study of problems concerned with the physiological function of the fat-soluble vitamine requires a food entirely devoid of it we have renewed the investigation from this standpoint. The protein, casein or edestin, was boiled three successive times with absolute alcohol under a reflux condenser for 1 hour, and then filtered off by suction. The commercial corn-starch used was treated in the same manner. The brewery yeast was first dried over night in an electric oven at 110°C. and then extracted in the same way.

In separate trials[13] we have assured ourselves that the yeast used as a source of water-soluble vitamine in all these tests does not lose its potency by desiccation at 110°C. followed by extraction with *absolute* alcohol. Rats have grown at a normal rate for 140 days to 336 gm. on rations in which such heated and alcohol-extracted yeast furnished the sole source of water-soluble vitamine in an otherwise adequate diet.

The curves of growth of a series of rats receiving a mixture of the extracted foods are given in Chart II. The diets consisted of extracted casein or edestin 18, extracted starch 48, salt mixture 4, lard 30 per cent, together with 0.4 gm. of extracted yeast daily.

[10] Osborne, T. B., and Campbell, G. F., *J. Am. Chem. Soc.*, 1900, xxii, 413. Osborne, T. B., and Wakeman, A. J., *J. Biol. Chem.*, 1916, xxviii, 1. van den Bergh, A. A. H., and Muller, P., *Proc. Acad. Sc. Amsterdam*, 1920, xxii, 748.

[11] McCollum, E. V., Simmonds, N., and Pitz, W., *Am. J. Physiol.*, 1916, xli, 363.

[12] Osborne, T. B., and Mendel, L. B., *Proc. Soc. Exp. Biol. and Med.*, 1918–19, xvi, 98; *J. Biol. Chem.*, 1920, xli, 549. See also Zilva, S. S., *Biochem. J.*, 1920, xiv, 494.

[13] The general methods of feeding, etc., employed in our investigations have recently been outlined by Ferry, E. L., *J. Lab. and Clin. Med.*, 1920, v, 735.

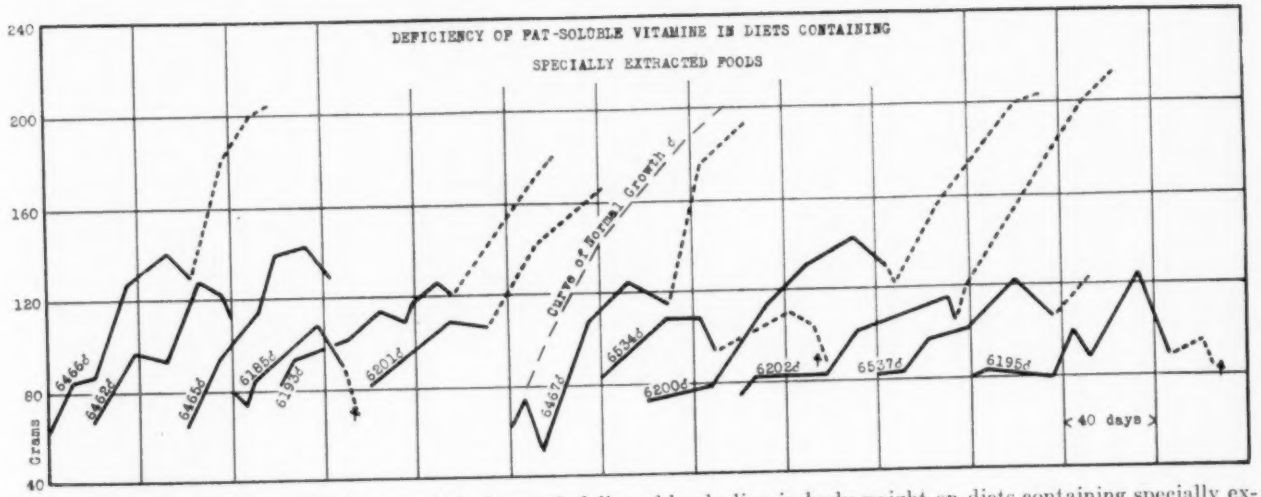

CHART II. Showing the preliminary period of growth followed by decline in body weight on diets containing specially extracted foods. During the periods represented by the interrupted lines renewal of growth was frequently secured by the addition of sources of fat-soluble vitamine to the diet. During the period represented by the uninterrupted line the food mixtures consisted of extracted casein or edestin 18, salt mixture 4, extracted starch 48, lard 30 per cent, together with 0.4 gm. of extracted yeast daily. Rats 6193, 6201, 6200, and 6202 developed the characteristic eye disease which was promptly cured after the administration of the fat-soluble vitamine.

These results should be contrasted with the records of experiments in which the foods were not specially extracted (Chart IV).

Since we have found[5] that the fat-soluble vitamine can be removed from butter fat by recrystallizing the latter from strong alcohol, commercial lard was melted and poured into absolute alcohol, heated to 60°C., cooled over night, and filtered by suction. This treatment was repeated three times, and the resulting solids were dried in a casserole over a steam bath. Experiments with this alcohol-extracted lard fed in place of the usual commercial product showed no essential differences in their outcome in so far as retardation of growth or well being were concerned.

It is a common experience that animals living on unsuitable diets tend to reduce their food intake. When the indispensable water-soluble vitamine is *incorporated* in such food the consumption of this food accessory may be greatly reduced because the animals do not eat enough of the rations containing it. A vicious circle is thus established whereby insufficient water-soluble vitamine leads to inadequate eating which in turn limits the quantity of both food and vitamine available. This criticism may be made of many investigations in which the undetermined and presumably lowered food intake occasions uncertainty regarding the supply of essential components other than the one specifically under investigation. It is especially true when the water-soluble vitamine is an integral component of the food mixture offered.

As an illustration of the difficulty of interpretation we may cite a series of unpublished experiments carried out by us several years ago with "fat-free" foods. The diets consisted of casein 18, salt mixture 4, yeast 2, and starch 76 per cent. Curves of body weight are shown in Chart III. It will be noted that on these rations supposedly devoid of fat-soluble vitamine the animals remained practically stationary in weight in contrast with what has been observed by us where lard was incorporated in the diet. All the rats scattered their food quite badly so that accurate estimates of the food intake could not be made. For this reason we are unable to conclude whether the failure to grow in the first period was due to inadequate intake of water-soluble vitamine or other essential nutrients, or was merely the expression of the *complete* lack of fat-soluble vitamine. All these animals began to grow when butter fat was added to the diet; but the food intake and consequently the intake of water-soluble

vitamine were simultaneously increased. McCollum[14] has published similar curves of complete failure to grow on a ration of casein, salts, agar-agar, and dextrin, carrying an extract of wheat embryo as a source of water-soluble vitamine, to which the criticism just applied to our own experiments may perhaps likewise be made.

The deleterious effect of a diet lacking in water-soluble (B) vitamine is far more acute in its manifestations than are the dietary failures observed by us when the fat-soluble vitamine is not supplied.[15]

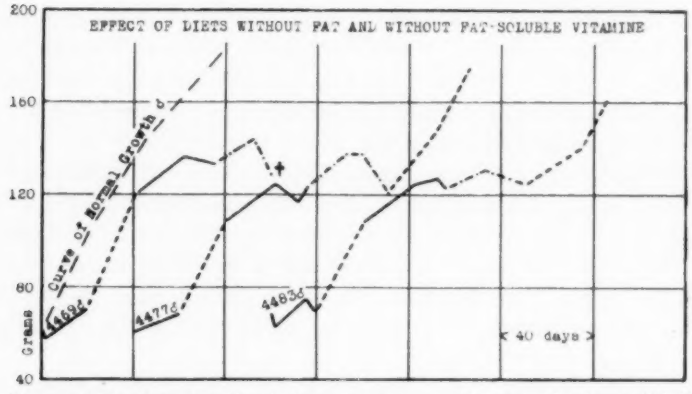

CHART III. Showing the failure to grow on fat-free foods (solid line) and on diets containing 9 per cent of lard (- - - - - -), with prompt growth whenever 9 per cent of butter fat (- - - - - -) was incorporated in the diet. The fat-free food mixture consisted of casein 18, salt mixture 4, starch 76, yeast 2 per cent.

In our newer series with alcohol-extracted foods this uncertainty has been averted by furnishing suitable daily doses of the water-soluble vitamine *apart* from the ration. Inasmuch as the animals greedily ate the separate allowance of yeast an adequate intake of this factor was assured. The outcome of the experiments with rations devoid of fat-soluble vitamine illustrated in Chart II has been a reduction of the period of undiminished

[14] McCollum, E. V., *Proc. Inst. Med.*, 1920, iii, 13, Chart I, Lot 417.

[15] Osborne, T. B., Wakeman, A. J., and Ferry, E. L., *J. Biol. Chem.*, 1919, xxxix, 35.

growth. However, even in these cases there was for some time a not negligible increment of weight. The prompt recovery of the animals tested, after their decline had begun, by the addition of butter fat, without any alteration in the dosage of water-soluble vitamine gave a clear answer to the nature of the deficiency in their previous diet. Whether the protein and starch used in our rations before they have been thoroughly extracted

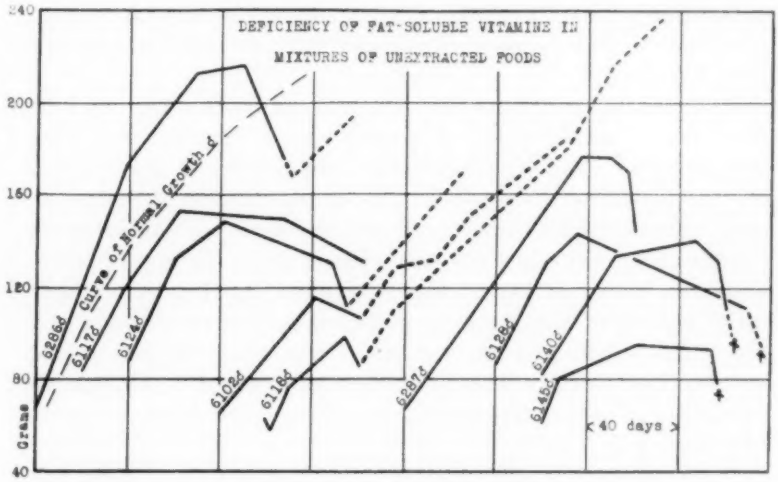

CHART IV. Showing preliminary growth followed by a decline in body weight on diets on (unextracted) foods deficient in fat-soluble vitamine, during the period indicated by the solid line. The recoveries during the periods indicated by the broken line follow the addition of butter fat as a source of fat-soluble vitamine to the diet. In the first period the food mixture consisted of casein or edestin 18, salt mixture 4, starch 48, lard 30 per cent, together with 0.4 gm. of yeast daily.

These records should be contrasted with the results of experiments in which the foods were specially extracted (Chart II).

with absolute alcohol are carriers of any noteworthy amount of fat-soluble vitamine may be judged by a comparison of the records just cited with those for a group of animals fed on precisely similar diets representing mixtures of food products which had not been extracted (Chart IV).

One gains the general impression that the extraction may

remove a small amount of the fat-soluble vitamine which if present would postpone the decline in weight somewhat longer. Steenbock and Gross[16] likewise give numerous records of a slight, though soon limited continuation of growth when a normal diet is replaced by one of highly purified food substances supposed to be free from fat-soluble vitamine. It should not be forgotten, however, that other substances which may have been removed by the extraction with alcohol may also be of importance for the well being of the animal. Convincing evidence that minute quantities of one or more lipoids are not essential has not yet been furnished.

It is interesting to note that whereas the fat-free diet used by McCollum,[14] which failed to permit growth, contained as the sole source of water-soluble vitamine the extract of only 11 gm. of wheat embryo, the precisely similar experiments of Steenbock and Gross, in which considerable initial growth was achieved on fat-free diets, contained the extract from 20 to 78 gm. of ether-extracted wheat embryo. Hence it may well be, as we have intimated, that the temporary growth on diets without any appreciable quantity of fat-soluble vitamine may be determined by the dosage of the water-soluble vitamine which the animals can secure. Indeed Steenbock and Gross have pointed out an instance

> " . . . . where presence of a subnormal amount of one vitamine was apparently brought to light by a similar situation with respect to another vitamine. When the deficiency of the water-soluble vitamine was corrected the amount of fat-soluble vitamine originally present was able to allow some further growth to result. . . . . It brings out one of the innumerable instances where a tendency to one nutritive deficiency heightens the susceptibility and results in the onset of symptoms resulting from similar or other unfavorable environmental conditions" (p. 507).

In the course of the feeding experiments it occurred to us that possibly the prolonged manipulations with alcohol render the food unpalatable. If this were true the nutritive failures might be due to undereating rather than to the lack of an essential ingredient removed by the extractions. This assumption was shown, however, to be untenable by a series of feeding trials in which the foods were treated with alcohol precisely as was the

[16] Steenbock, H., and Gross, E. G., *J. Biol. Chem.*, 1919, xl, 501.

case for the extracted foods. The products were subsequently dried to drive off the alcohol without filtration or removal of any non-volatile ingredient. Chart V shows the outcome of feeding trials with such foods. Since where butter fat was present growth was essentially normal the assumption which has found its way into the literature that heating with alcohol deteriorates the nutritive value of casein is disposed of. Where lard was the only fat present the curves of growth are not essentially unlike those

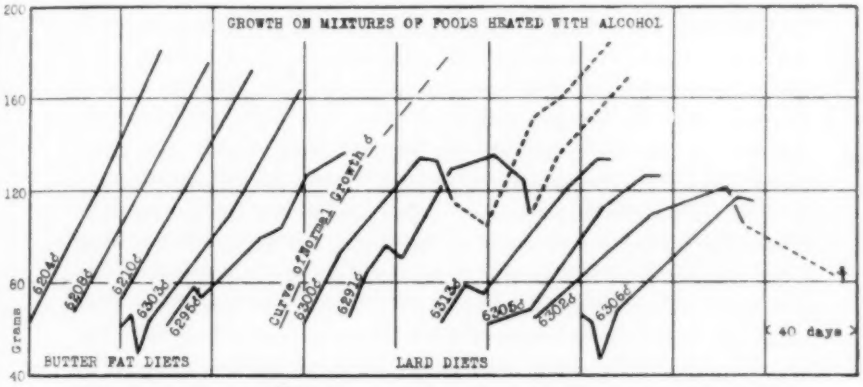

CHART V. Showing the absence of noteworthy effect from heating with alcohol in the case of mixtures of foods either adequate for growth (Rats 6204, 6208, 6210, 6303, 6295) or deficient in fat-soluble vitamine (Rats 6291, 6300, 6302, 6313, 6305, 6306). The food during the periods indicated by the solid lines consisted of casein (heated with alcohol) 18, starch (heated with alcohol) 48, salt mixture 4, butter fat 9 or 0, lard 21 or 30 per cent, with 0.4 gm. of alcohol-extracted yeast daily. During the periods indicated by the broken line butter fat was supplied as a source of fat-soluble vitamine.

for the similar experiments with unextracted foods represented in Chart IV.

In the successful feeding experiments with the lard-containing foods reported by Daniels and Loughlin, to which reference was made in our opening paragraph, it is reported that in order to reduce the content of fat-soluble vitamine to a minimum in the experimental rations " . . . . both the casein and the wheat embryo, previous to the alcohol treatment, were extracted for 48 hours with ether ('Squibb, for anæsthesia') in a Soxhlet

apparatus. The lard used was a commercial product rendered from the leaves and back fat." Must we assume for the present either that the lard from different sources varies in its content of fat-soluble vitamine or that ether alone fails to extract it from the non-fat foodstuffs?

It has been alleged that the ability of animals to grow for some time in the supposed absence of fat-soluble vitamine is due to "exceptional vitality" of the individuals or to reserve stores of the vitamine in the body.[17] Unless we are to accept some indefinite explanation of this sort, it seems necessary to conclude, in the light of our experience, that removal of the fat-soluble vitamine from even purified proteins and carbohydrates is accomplished with far greater difficulty than has been hitherto suspected. An entirely convincing crucial experiment, in which nutritive failure immediately ensues upon the administration of diets fully adequate *in every respect* except for the presence of fat-soluble vitamine, remains to be made. It is significant that older rats thrive for a longer time than do the younger ones on the same diets nearly if not entirely free from the fat-soluble vitamine. This is in contrast with the well established fact that at all periods the lack of water-soluble vitamine is speedily manifested.

[17] McCollum, E. V., and Davis, M., *J. Biol. Chem.*, 1915, xx, 641. Also Drummond, J. C., *Biochem. J.*, 1919, xiii, 81, who states that occasionally rats are encountered which "exhibit a power to grow for long periods of time although receiving a diet seriously deficient in fat-soluble A."

COMPARATIVE METABOLISM OF PROTEINS OF UNLIKE COMPOSITION.*

By WALTER G. KARR.

With the Cooperation of Edward Tolstoi.

(From the Sheffield Laboratory of Physiological Chemistry, Yale University, New Haven.)

(Received for publication, November 12, 1920.)

Proteins contain nitrogen in different groupings; *e.g.*, amino nitrogen, amide nitrogen, glyoxaline and proline, and the guanidine rest. On hydrolysis of proteins with strong mineral acids an ammonia fraction is very easily split off and represents the amide nitrogen in the molecule. This ammonia fraction runs parallel with the amount of dicarboxylic acids—aspartic and glutaminic—from the amide nitrogen of which it is probably for the most part derived (Osborne, 1909).

The proportion of these unlike types of nitrogenous groups varies in different proteins; *e.g.*, casein has a low content of amide nitrogen while the proteins of the wheat kernel are richer in this grouping of nitrogen. A sample of commercial casein containing 12.6 per cent total nitrogen, hydrolyzed for 2 hours with 20 per cent HCl, yielded 10.5 per cent of its nitrogen as ammonia nitrogen while a sample of gluten having 13.0 per cent total nitrogen gave 21.6 per cent of its nitrogen as ammonia nitrogen under the same conditions.

We know that the proportions of the end-products of nitrogenous metabolism in the urine, *e.g.* urea N, ammonia N, creatinine N, creatine N, etc., may be varied by the nature of the diet. It is not definitely ascertained, however, whether this partition

* The data in this paper are taken from the writer's dissertation presented in partial fulfillment of the requirements for the degree of Doctor of Philosophy, Yale University, 1920. A part of the expenses of the research were defrayed by a contribution from the Russell H. Chittenden Fund for Research in Physiological Chemistry.

of nitrogen can be altered by the type of distribution of nitrogen in the protein intake; *i.e.*, whether amide nitrogen has the same fate in metabolism as amino nitrogen. Therefore the following experiments on the metabolism of different diets varying only with respect to the character of the protein therein have been undertaken. Inasmuch as the character of the metabolic change may be determined by the plane of nutrition, *i.e.* whether an animal is on a low nitrogen intake barely sufficient for its needs, or on a high intake, experiments were conducted at different levels of protein intake.

Three female dogs were employed. They were kept in ordinary metabolism cages and catheterized at 24 hour intervals under conditions to preclude infection. Experimental periods lasted 4 or 5 days each. The feces were marked into periods with carmine. They were dried on the water bath with alcohol to which a few drops of acid were added. Water was given *ad libitum*. Total nitrogen was determined by the Kjeldahl method; ammonia nitrogen by the Folin aeration method; creatinine by the Folin micro method; creatine by the same procedure after treatment with 2 cc. of normal HCl in an autoclave for 20 minutes at 20 pounds pressure; phosphates by titration with uranyl acetate; hydrogen ion concentration by the Sörensen colorimetric method using sodium alizarin sulfonate as indicator for the range pH 5.0 to 6.0 and neutral red for pH 6.0 to 7.5.

The proteins fed were casein and wheat gluten. The casein[1] was a light yellow commercial preparation, finely ground, containing about 12.7 per cent of nitrogen. The gluten was a commercial variety,[2] very finely ground, containing 12 to 14 per cent of nitrogen. In addition to the protein under investigation the remaining calorific requirement was supplied by approximately equal weights of lard and sucrose to which were added 2 gm. of NaCl and 5 gm. of bone ash, the latter to give proper consistency to the feces. The diets with a few exceptions were fed in one meal soon after catheterization in the morning and were made to supply about 70 calories per kilo of body weight, but were increased in certain experiments (see Table I).

[1] Casein was kindly supplied by Lister Brothers, New York.

[2] Wheat gluten was purchased from Menley and James, New York, under the commercial name Glidine.

TABLE I.

Results of Feeding Experiment with Casein and Gluten.

(The data give daily averages of the periods.)

Experiment No.	N intake.	Food intake.	Urine.							
			Volume.	Reaction.	Total N.	NH_3 N.	$\frac{NH_3\ N}{Total\ N}$	Creatinine N.	Creatine N.	Phosphorus.
	gm.	*calories*	*cc.*	*pH*	*gm.*	*mg.*	*per cent*	*mg.*	*mg.*	*mg.*
1. Casein period, 5 days; Dog B, weight 8.4 kg.	20.2	720	500	6.1	17.3	1,140	6.6	78	*	944
2. Gluten period, 5 days; Dog B, weight 8.4 kg.	19.9	600	590	6.4	17.8	967	5.4	78	*	164
3. Gluten period, 3 days; Dog A, weight 9.7 kg.	19.9	600	470	6.4	17.3	850	5.0	95	*	140
4. Gluten period, 5 days; Dog A,† weight 10.7 kg.	13.0	900	450	6.5	11.3	640	5.7	96	2	213
5. Gluten period, 3 days; Dog C, weight 6.5 kg.	14.9	460	445	6.4	13.2	724	5.5	55	2	
6. Casein period, 5 days; Dog C, weight 6.5 kg.	6.95	440	260	6.1	6.15	406	6.6	58	16	352
7. Gluten period, 5 days; Dog C, weight 6.5 kg.	6.82	430	330	6.1	6.76	345	5.4	57	12	125
8. Casein period, 5 days; Dog A,† weight 10.2 kg.	6.32	700	220	6.1	5.21	350	6.7	100	0	375
9. Gluten period, 5 days; Dog A,† weight 10.4 kg.	6.40	670	370	6.6	5.84	352	6.0	99	3	211

* When creatine was determined on urines from dogs on a very high protein diet the combined creatinine after HCl treatment was always a few milligrams less than the preformed creatinine. The point was not further investigated except that a few normal human urines were simultaneously analyzed and did not show the phenomenon, the combined and preformed creatinine being identical.

† The dog was pregnant.

TABLE I—*Concluded.*

Experiment No.	N intake.	Food intake.	Urine.							
			Volume.	Reaction.	Total N.	NH_3 N.	$\frac{NH_3\ N}{Total\ N}$	Creatinine N.	Creatine N.	Phosphorus.
	gm.	*calories*	*cc.*	*pH*	*gm.*	*mg.*	*per cent*	*mg.*	*mg.*	*mg.*
10. Casein period, 5 days; Dog B, weight 8.2 kg.	6.57	600	260	6.1	5.72	380	6.6	78	5	442
11. Casein period, 5 days; Dog B, weight 8.6 kg.	2.94	610	220	6.0	2.77	195	7.1	82	5	280
12. Gluten period, 5 days; Dog B, weight 8.5 kg.	2.98	600	210	6.3	3.03	209	6.9	82	4	206
13. Casein period, 5 days; Dog C, weight 6.7 kg.	2.56	470	200	6.2	2.20	112	5.2	59	25	227
14. Gluten period, 5 days; Dog C, weight 6.5 kg.	2.57	460	200	6.3	2.32	117	5.0	59	14	164

DISCUSSION.

The urinary ammonia output is not influenced to any appreciable extent by the character or quantity of the two so widely different proteins in the diet. If the metabolism of the abundant amide nitrogen of the wheat proteins were different from that of the amino nitrogen of the casein one might anticipate that more urinary nitrogen in the form of ammonia would be excreted when the diet contained the wheat gluten. This was not the case and in general the excretion was a little lower. The average excretion on the casein diets was 6.5 per cent of the total nitrogen and 5.6 per cent on the gluten diets. The slight increase in the hydrogen ion concentration and the urinary ammonia on the casein diet might easily be explained by the greater potential acidity of the casein. The greater acidity of the casein is apparently mostly taken care of by the phosphate regulation. The creatinine output was evidently entirely of endogenous origin. The creatine found seems independent of the nature of the protein.

Additional Experiments with Yeast Protein.

Experiments similar to the preceding were conducted with yeast as the sole source of nitrogen in order to study the partition of the urinary nitrogen and to compare it with that following the ingestion of the other two proteins, and incidentally to determine the utilization of the yeast nitrogen. The literature shows rather conflicting statements as to the availability of the yeast protein in the animal body.

Völtz (1905) reported 83.6 per cent of nitrogen utilized in one experiment with a dog. Völtz and Baudrexel (1911) reported an experiment on a man in which about one-half the nitrogen was supplied by yeast; 86 per cent was utilized. Rubner (1916) giving 13.7 gm. of yeast nitrogen plus 30.9 gm. of meat nitrogen to a dog calculated that 98.4 per cent of the yeast nitrogen was utilized. Deutschland (1917) gave a dog a diet containing about one-half its nitrogen as yeast nitrogen and obtained utilizations of 83.5 and 89 per cents. Schill (1918) reports coefficients of 40 per cent in one case and 75 per cent in the other. The above data were obtained by using yeast as a part only of the nitrogen intake and obviously do not give a clear idea as to the utilization of the yeast nitrogen. Funk, Lyle, and McCaskey (1916) in four experiments on man in which yeast supplied the principal sources of nitrogen found 70 to 80 per cent utilized and decided that it "cannot very well be recommended as a sole protein source." Schill (1918) reported two experiments where yeast was the only source of nitrogen in the diet of two dogs. The intakes were low (1.75 and 1.54 gm. of nitrogen per day); the utilization was 66 and 76 per cent respectively.

The yeast used in the present investigation was a dried commercial brewery yeast.[3] It contained nitrogen 7.2, moisture 9.1, and ash 6.6 per cent. Neuberg (1915) estimated that 46 to 48 per cent of the nitrogen of yeast is protein nitrogen while Bokorny (1915) estimated it at 60 per cent. The yeast was suspended easily in water and was readily given with the other constituents of the diet each day through a stomach sound. With this exception the experiments were conducted similarly to those reported above (see Table II).

The ammonia nitrogen output compares favorably with that after feeding casein and gluten, giving further evidence that it is not influenced by the character of the protein. On certain days of the lower nitrogen intake periods the ammonia was compara-

[3] Supplied by Hinckle Brewery, Albany.

TABLE II.

Results of Feeding Experiments with Yeast.

(The data give daily averages of the periods.)

Experiment No.	Yeast.	N intake.	Food intake.	Urine.								Feces.			N balance.	N utilization.
				Volume.	Reaction.	Total N.	NH_3 N.	$\frac{NH_3\ N}{Total\ N}$	Creatinine N.	Creatine N.	Phosphorus.	Weight.	N			
	gm.	gm.	calories	cc.	pH	gm.	mg.	per cent	mg.	mg.	mg.	gm.	per cent	gm.	gm.	per cent
1. 5 days; Dog B, weight 8.6 kg.	90	6.52	625	560	6.5	5.27	345	6.5	84	6	972	38	4.0	1.5	−0.3	77
2. 5 days; Dog C, weight 6.4 kg.	70	5.00	460	360	6.5	3.90	240	6.2	58	30	700	32	4.0	1.3	−0.2	75
3. 5 days; Dog B, weight 8.5 kg.	40	2.90	610	345		2.29	213	9.3	81	5	510	21	3.0	0.6	0.0	79
4. 5 days; Dog C, weight 6.6 kg.	35	2.53	480	400		2.01	169	8.4	59	32	506	14	3.1	0.4	+0.1	82

tively high, but no explanation is evident until more is known concerning the nature of the nitrogenous compounds of yeast. The excretion of urinary phosphorus is high as would be expected from the high content of nucleic acid in the yeast. The nitrogen balance and percentage utilization indicate that yeast has about 80 per cent of its nitrogen fairly efficient for nutrition.

CONCLUSIONS.

As far as is indicated by the urinary nitrogenous end-products, the metabolism of two proteins, casein and wheat gluten, widely different in their chemical make-up, is essentially the same.

Despite the great diversity in amide nitrogen in the proteins compared, the relative excretion of urinary ammonia was approximately the same.

In metabolism experiments with comparable quantities of yeast as the sole source of protein, the partition of urinary nitrogen compared favorably with what was observed after feeding the other two proteins.

The utilization of the yeast nitrogen was about 80 per cent.

The writer wishes to thank Professor Lafayette B. Mendel for his suggestion of the problem and advice throughout the course of the investigation.

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RIGOR MORTIS IN SMOOTH MUSCLE AND A CHEMICAL ANALYSIS OF FIBROMYOMA TISSUE.

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(Received for publication, August 30, 1920.)

1. Experiments Demonstrating a Postoperative Rigor in Fibromyomas.

Studies of rigor mortis recorded in the literature have been made almost exclusively on skeletal muscle, and whatever conclusions there may be regarding its occurrence in smooth muscle seem to have been reached largely by inference from such studies, rather than by actual experimental observations. Wells[1] states that all forms of muscle, striped, smooth, and cardiac, undergo rigor mortis, manifested by a shortening and thickening. Saxl,[2] investigating the distribution of muscle proteins in bovine uteri, observed no difference between the analyses of tissues fresh and after 24 hours. He did not see a postmortem rigidity of these uteri. Nasse, according to this investigator, found the intestinal wall firmer several hours after death than immediately post mortem, and concluded that this is a rigor mortis contraction of the intestinal musculature. Hawk,[3] referring to Saxl's study, says there is a difference of opinion regarding the occurrence of true rigor in non-striated (smooth) muscle. Such statements in the literature are sufficiently at variance with the observed contraction and intussusception of bowel post mortem, and to similar or postoperative rigidity of uteri and fibromyomas to deserve investigation.

[1] Wells, H. G., Chemical pathology, Philadelphia, 3rd edition, 1918.

[2] Saxl, P., *Beitr. chem. Physiol. u. Path.*, 1907, ix, 1.

[3] Hawk, B. P., Practical physiological chemistry, Philadelphia, 6th edition, 1918.

Contraction of bowel segments or their intussusception has been observed post mortem, no doubt, by every pathologist, but the doughy consistency of a uterus or a fibromyoma changing to a rigid firmness soon after death or surgical removal is not so commonly appreciated. Histologically, fibromyoma tissue contains much smooth muscle, and such contractile phenomena as it may manifest are due, no doubt, to this element of its structure.

In order to study the rigidity mentioned, and, if possible, correlate it with the physical changes of skeletal muscle known as rigor mortis, sections of operatively removed uteri and fibromyomas 1 cm. square and 4 cm. long were mounted soon after

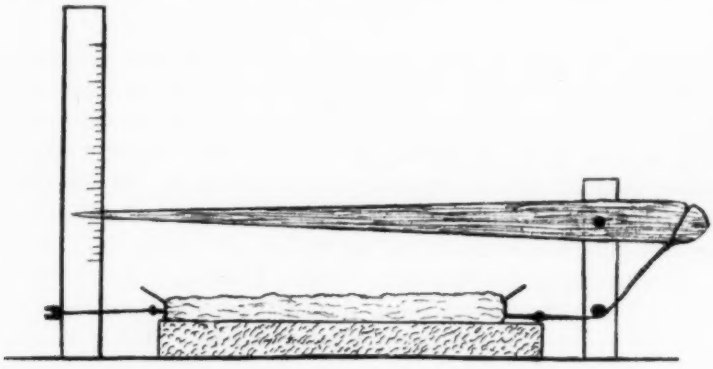

Fig. 1. Apparatus for mounting muscle.

removal, as shown in Fig. 1. The tissue preparation was placed in a moist chamber at room temperature (62–70° F.), moistened with a few drops of normal salt solution, and the contractions were observed.

As soon as the fresh tissue preparation is mounted, contraction begins and continues gradually to a maximum. In order to represent this change graphically, a curve was made by plotting along the ordinate the contraction measured on the millimeter scale of the apparatus, and along the abscissa the time in minutes. Two such curves are given in Fig. 2, one of them for uterine muscle, the other for fibromyoma tissue. After the period of contractility has passed, which may be as short as 1 hour after removal, forceful extension is not followed by shortening.

All the experiments agree in result with the graphs prepared, excepting such fibromyoma tissue as has undergone retrogressive changes known as "red degeneration." Such tissue manifests none of the contractile phenomena observed with other fibromyomas and uteri.

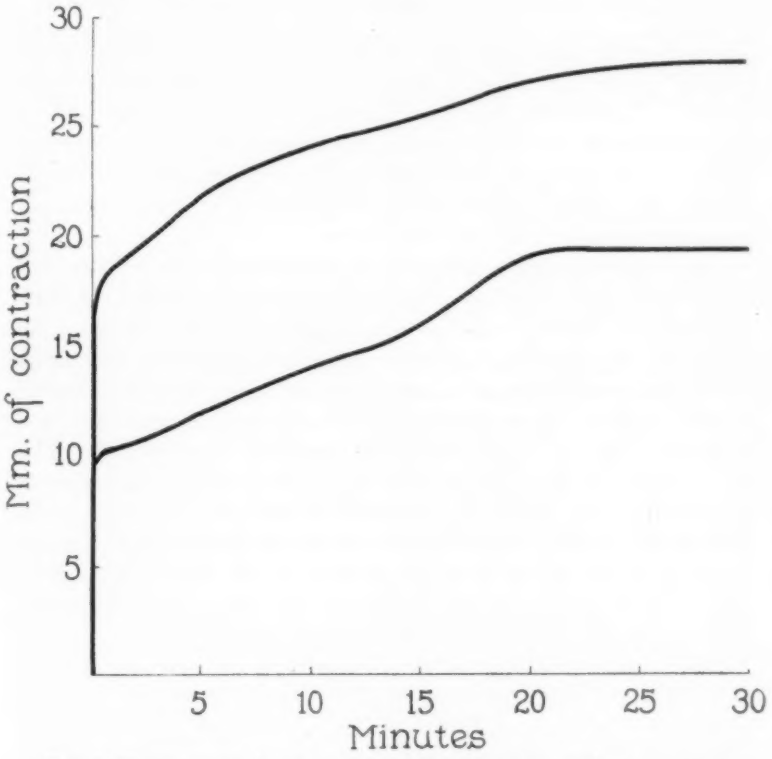

FIG. 2. Upper curve shows contraction of fibromyoma; lower curve contraction of uterus.

It is possible to say, from these observations, that a postoperative rigor occurs in smooth muscle, which is similar, at least in so far as contraction is concerned, to the postmortem rigor of striated muscle.

The heart, in postmortem examinations generally, is said to be in firm systole or to have ceased action in systolic contraction.

This statement, no doubt, is incorrect, for the postmortem rigor of heart muscle is similar to that observed with other involuntary muscle.

2. Experiments Determining a Postoperative Acidity in Fibromyomas.

Postmortem rigor and rigor induced under other conditions in skeletal muscle are known generally to be accompanied by an acidity. Lactic acid has been identified in such tissues and is believed largely to be the acid causing this change in reaction. With the exception of certain heat rigor experiments on smooth muscle by Meigs,[4] all these observations have been made on skeletal muscle.

Since a postoperative rigor can be demonstrated in fibromyoma tissue and uteri, it is possible that an acidity similar to that occurring in striated muscle may accompany this change. To determine it, standard solutions and the appropriate indicators in solution were prepared carefully according to Clark and Lubs.[5] Triple distilled "conductivity water" was used in making these solutions and in conducting the reaction experiments. The tissue reaction was determined by placing 10 gm. samples of fibromyoma and uterine tissue in 15 cc. of water, and after sufficient time (usually a minimum of 15 minutes was taken) for the reaction of the water to approach that of the tissue, 2 cc. were placed in a comparing tube, colored with the proper indicator, and compared with a series of standard solutions.

The results of the individual experiments are given in Table I.

While the contention is not made that the hydrogen ion concentration obtained by the method described represents the maximum acidity, for doubtless dilution of the tissue fluids and other factors must be considered, yet these tests demonstrate uniformly an acidity developing rapidly in fibromyoma and uterine tissue manifesting a postoperative rigor. This change in reaction is another similarity correlating the rigor of fibromyomas (non-striated muscle) with the postmortem rigor of skeletal muscle. The only exception again is the fibromyoma

[4] Meigs, E. B., *J. Biol. Chem.*, 1909, vi, p. xviii.

[5] Clark, W. M., and Lubs, H. A., *J. Bact.*, 1917, ii, 1, 191.

tissue with changes known as "red degeneration" in which neither an acidity nor a rigor developed. The substance giving the acid reaction to the tissues may be lactic acid, for several of the fluids in which the fibromyoma tissue had been contained reacted positively with the ferric chloride test for this acid.

Kondo[6] determined quantitatively the increase of lactic acid in expressed skeletal muscle fluid, and found that its formation is unusually rapid, the maximum concentration in some fluids having been reached within 30 minutes. He observed that

TABLE I.

Hrs.	1	2	3	4	5	6	7	22	23	24
	pH	*pH*	*pH*	*pH*	*pH*	*pH*	*pH*	*pH*	*pH*	*pH*
Fibromyoma				6.8	6.8	6.8		6.2		6.0
"									6.0	
"	6.4			6.4			6.2	6.2		6.2
Uterus	6.4									
Fibromyoma.*	7.0			7.2		7.2	7.2			
" †			6.6							
"			6.2	6.0		6.0			6.0	
"			6.0							

* Red fibromyoma, did not contract in moist chamber.
† " " contracted little " " "
Some fluids in these experiments reacted with the ferric chloride test for lactic acid.

lactic acid production is completely inhibited by the addition of acid (N H_2SO_4) to the muscle fluid, and is favored by the addition of sodium bicarbonate, there being an optimum concentration of this alkali in such fluids beyond which it exerts no influence. In the presence of a small quantity of alkali in the expressed muscle fluid, the formation of lactic acid soon reaches a maximum, and is inhibited at a certain hydrogen ion concentration. This concentration is not given by Kondo. His conclusion, however, is supported by the results of this study for there has been found a fairly uniform maximum (pH 6.0) which the usual tissue acidity does not exceed.

[6] Kondo, K., *Biochem. Z.*, 1912, xlv, 63.

3. Postoperative Alkali Reserve Changes in Fibromyoma Tissue.

To study further the postoperative change in the reaction of these tumors, 3 cc. of fluid were expressed from small pieces of tissue with a hand meat press. The fluids were equilibrated with air containing 5.5 per cent carbon dioxide and analyzed according to the Van Slyke and Cullen[7] method, the proper corrections for 0°C. and 760 mm. barometric pressure being made.

Two such determinations were made with fibromyoma tissue 24 hours after removal, the carbon dioxide capacities of their fluids being respectively 3.93 and 6.06 volumes per cent; the fluid of a third after 8 hours, 8.4 volumes per cent; and that of

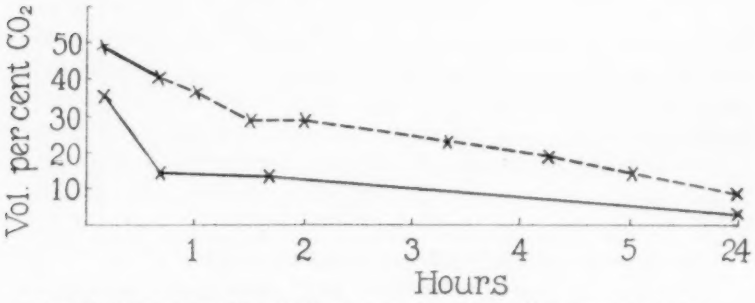

FIG. 3. Postoperative alkali reserve changes in fibromyoma tissue.

another after 48 hours, 6.56 volumes per cent. The last mentioned determination was made after the postoperative rigor had disappeared.

The rapidity with which this change in the alkali reserve occurs was determined then by testing at short intervals other fibromyomas obtained immediately after their removal. The results of two such determinations are shown in Fig. 3.

These curves and the results mentioned otherwise support those results obtained in the experiments made for determining the postoperative variation of the hydrogen ion concentration, in that they demonstrate a marked and rapid loss of the tissue alkali reserve. The curves, especially, graphically represent the speed with which this change occurs.

[7] Van Slyke, D. D., and Cullen, G. E., *J. Biol. Chem.*, 1917, xxx, 289.

4. The Reducing Carbohydrate Content of Fibromyoma Tissue.

The possibility that some carbohydrate may be the substance from which an acid or acids arise in the tissues during postoperative rigor suggested investigating the reducing sugar content of fibromyoma tissue at varying short intervals of time. To do this, the fluid of recently removed fibromyomas was expressed from small pieces of tissue with a hand meat press, a specimen as free from blood as possible being taken. Duplicate 2 cc. samples were diluted with 14 cc. of distilled water and the protein was precipitated according to the Folin and Wu[8] technique by adding 2 cc. of 10 per cent sodium tungstate solution and 2 cc. of $\frac{2}{3}$ N sulfuric acid. The precipitated proteins were filtered off, and 2 cc. of the clear filtrate used for making the sugar determinations according to the recent method of Folin and Wu[9] with the special tubes recommended. All determinations were made in duplicate on the duplicate samples of fibromyoma fluid, and the reducing power of the clear filtrate was estimated in milligrams of dextrose.

Three separate samples of fibromyoma fluid taken 24 hours after operative removal contained per cc. the equivalent of 0.2650, 0.3535, and 0.3312 mg. of dextrose. A uterus examined 24 hours after surgical removal contained three fibromyomas with values of 0.333, 0.211, and 0.198 mg. of dextrose per cc. of expressed fluid. The values for other fibromyoma tissues are charted in Fig. 4.

These curves disclose a rapid diminution of reducing carbohydrates in fibromyoma tissue, especially during the 1st hour after removal, but continuing slowly thereafter. It is not likely that these substances disappear completely, for appreciable amounts are present 24 hours after removal.

Although the experiments mentioned do not demonstrate necessarily a carbohydate origin of the tissue acidity, there is a certain parallel between the rate of acid increase, the rate of alkali reserve decrease, and the rate of reducing carbohydrate diminution, each rapidly attaining a maximum at about the same time and continuing thereafter at an approximate level.

[8] Folin, O., and Wu, H., *J. Biol. Chem.*, 1919, xxxviii, 81.
[9] Folin, O., and Wu, H., *J. Biol. Chem.*, 1920, xli, 367.

Of interest in correlating this parallelism are the comments by Haggard and Henderson[10] on asphyxial acidosis. Araki's[11] observation that partial asphyxia causes the appearance of lactic acid (lactates) in the urine has been interpreted, according to these investigators, to indicate that in the absence of adequate oxygen supply an acidosis develops. This interpretation of Araki's observations, they continue, has gained support from the

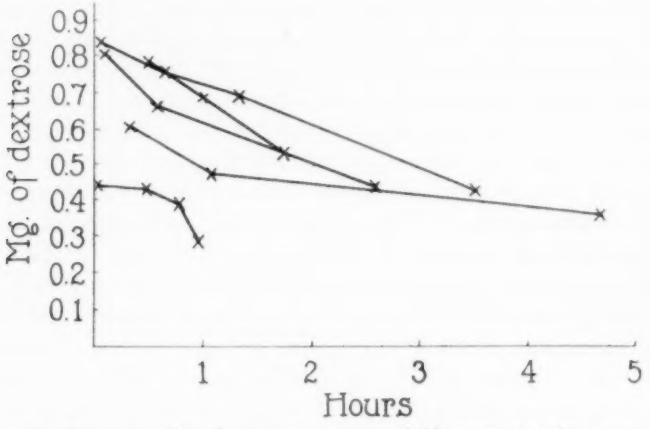

FIG. 4. Postoperative dextrose content of fibromyoma tissues per cc. of tissue fluid.

fact, now apparently well demonstrated, that the first step chemically in the production of energy from carbohydrates in the animal body is anaerobic; sugar breaking down into lactic acid, which then is oxidized to carbon dioxide, the latter reaction being secondary, more a process of elimination than one of energy production. Embden, Baldes, and Schmitz,[12] among others, have discussed the mechanism of lactic acid formation from glucose in the animal body.

[10] Haggard, H. W., and Henderson, Y., *J. Biol. Chem.*, 1920, xliii, 3.
[11] Araki, T., *Z. physiol. Chem.*, 1894, xix, 422.
[12] Embden, G., Baldes, K., and Schmitz, E., *Biochem. Z.*, 1912, xlv, 108.

5. Chemical Analysis of Fibromyoma Tissue.

As part of the study of rigor mortis in fibromyomas, a chemical analysis of this tumor tissue was made. Samples were ground finely, weighed by difference, and preserved in redistilled 95 per cent alcohol sufficient to make approximately 75 per cent concentration until the alcohol-ether extraction in a Soxhlet

TABLE II.

	per cent
Alcohol-ether residue	16.44
" extract (dried)	3.38
Total solids	19.82
Water	80.18

Alcohol-ether residue per gm. solids.

	mg.
Total protein	792.0
Protein sulfur	4.6
" phosphorus	1.5

Alcohol-ether extract.

	Extractives.	Lipins.
	mg.	*mg.*
Total inorganic (ash)	30.2	
" solids	101.3	
" sulfur	0.7	0.5
Inorganic sulfur	Trace.	
Total phosphorus	2.3	2.2
Inorganic phosphorus	1.3	
Total nitrogen	4.5	2.3

extractor was made. The tissue analysis was made according to standard methods, the sulfur and phosphorus being determined gravimetrically as barium sulfate and magnesium pyrophosphate respectively, and the nitrogen by the Kjeldahl method. The results are given in Table II.

The proportion of total solids and water in the fibromyoma tissue analyzed approximates those found in the castration granuloma of swine,[13] and in many other respects the analyses of the alcohol-ether-soluble and alcohol-ether-insoluble fractions of each correspond closely.

[13] Hirsch, E. F., and Wells, H. G., *Am. J. Med. Sc.*, 1920, clix, 356.

CONCLUSIONS.

There is a postoperative (postmortem) rigor of smooth muscle which is manifested by changes similar to those known in skeletal muscle as rigor mortis. Each variety of muscle in this change contracts, and is accompanied by a distinctly increased tissue acidity. With smooth muscle this acidity develops rapidly to a maximum, and is paralleled closely by a rapid diminution of its reducing carbohydrates, and by a decrease of its alkali reserve. There is a fairly uniform maximum (pH 6.0) tissue acidity in rigor mortis of fibromyomas that in part, at least, is due to lactic acid.

A chemical analysis of fibromyoma tissue is given.

THE CULTIVATION OF YEAST IN SOLUTIONS OF PURIFIED NUTRIENTS.

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There has existed since the controversy between Pasteur and von Liebig (1, 2, 3) and the subsequent work of Mayer (4) and von Nägeli (5) a belief that yeast in nutrient solutions affording only ammonium salts as a source of nitrogen failed to multiply to an appreciable extent. The addition of small quantities of impurities of certain kinds, especially yeast extract, exerted a profound effect on the growth of yeast cells in such media. The nature of the substance or substances which affect the multiplication of yeast cells in so marked a degree has never been clearly stated. To define it Wildiers (6) coined the term "bios." Yeast itself was considered the best source of this growth-promoting substance. Very small amounts of "bios" were needed to insure rapid proliferation, and it was thought that the better growth secured in nutrient solutions of purified foodstuffs in which an ammonium salt served as the sole source of nitrogen when a large seeding of yeast was made, as contrasted with the results when one or very few cells were cultivated, was due to the death and autolysis of some of the cells. These dead cells contributed the hypothetical "bios" to the solution, and thus enabled the living ones to proliferate.

The proofs brought forward in support of this view have not been entirely convincing, but have been widely accepted. That the acceptance has not been universal is made evident by the numerous papers that are still being written in support of or in opposition to it. Quite recently it has been suggested that the "bios" of Wildiers is perhaps identical with the antineuritic substance concerned in the nutrition of the higher animals. Williams (7) and Bachmann (8) have devised procedures for the detection

and approximate estimation of the antineuritic substance by the use of yeast as a test organism. Their methods are based on the assumption that there exists a definite requirement by yeast of the antineuritic substance which has generally been assumed to be identical with the dietary factor water-soluble B. These authors hold the view that they have demonstrated experimentally that the multiplication of yeast cells in a solution containing pure chemical substances, all of which can be named, and the fermentative power of the yeast are quantitatively influenced by the addition of substances known to contain water-soluble B (7, 8). In a recent paper from this laboratory (9) it has been shown that extracts of certain natural foods, *e.g.* wheat germ, rolled oats, muscle tissue, etc., even when treated so as to contain so little of the antineuritic substance that its presence cannot be demonstrated experimentally with rats, still exert a profound influence on the proliferation of yeast cells. The same is true of the addition of mixtures of amino-acids and of glucose. The view that the growth of yeast can be made a quantitative method for the dietary factor water-soluble B must be regarded as questionable until supported by more convincing experimental proof.

The following experimental work was undertaken in order to throw further light on the problem of the requirement of yeast of the dietary factor water-soluble B. The problem might also be stated, because of the nature of the experimental work and the line of reasoning involved, to be a test of the identity of the antineuritic substance water-soluble B with "bios."

We were successful in securing the multiplication of yeast in fifteen successive seedings in nutrient solutions of purified chemical substances.

In order to have a synthetic solution free from any trace of water-soluble B it seemed expedient to restrict the nutrient substances as nearly as possible to those of mineral origin. More than half a century of experimentation on the nutrition of yeast furnished abundant material for references. According to Lafar (10) yeast may under proper conditions make excellent use of ammoniacal nitrogen. Enough experimental evidence in favor of at least a partial utilization of an inorganic nitrogen supply by yeast cells was found to warrant the use of ammonium sulfate as the source of nitrogen. The sucrose used as the carbohydrate supply

was prepared from the best granulated cane sugar by repeated precipitation from a concentrated water solution by absolute alcohol.

Nutrient Solution 1. 2 Per Cent Sucrose.

1 liter distilled water.
20 gm. sucrose, recrystallized.
3 " ammonium sulfate, C.P.
2 " potassium dihydrogen phosphate, C.P.
0.25 gm. calcium chloride, C.P.
0.25 " magnesium sulfate, C.P.

Nutrient Solution 2. 5 Per Cent Sucrose.

The second nutrient solution differed from the first only in that it contained 50 gm. of sucrose per liter.

The solutions were heated to boiling on 2 successive days. On the 3rd day portions of 25 cc. were measured with a sterile pipette into a number of small sterile Erlenmeyer flasks, which were then heated to boiling, stored in a cupboard, and kept free from possibilities of contamination.

Three strains of yeast, baker's yeasts "F" and "XII" and brewer's yeast "K," were obtained from the Fleishmann Laboratories. A small loopful of these pure cultures was used to seed 25 cc. of sterile nutrient solution. The flasks were then well shaken to distribute the cells and allowed to stand undisturbed in the cupboard at room temperature. There was not enough yeast to produce visible turbidity in the solutions. At the end of a week there was a very perceptible growth of yeast on the bottom of the flasks and the solutions showed considerble turbidity when shaken. 1 cc. of the well agitated suspension in Flask A was transferred to a second seeding, Flask B, which was kept for a week, when the turbidity seemed about the same as in Flask A 1 week after seeding. A third seeding was made from Flask B to Flask C. This successive seeding was continued to the fifteenth seeding with no apparent change in the rate of growth or diminution in the fermentative activity of the yeast. Every precaution was taken to insure pure seedings, a microscopic examination of a sample from each flask being made before seeding the next flask, because of the known influence of other fungi (11, 12).

The seedings in Nutrient Solution 2 were conducted in the same way as those in Nutrient Solution 1. It was, however, possible to make seedings every 5 days rather than every 7 days as in the first series. It was noted in both series that the two strains of baker's yeast, "F" and "XII," made a somewhat better growth than brewer's yeast "K."

Flask A containing 25 cc. of nutrient solution had added to it the "bios" contained in one loopful of a pure culture of yeast cells. If these cells gained no "bios" and lost none, Flask B received 1/25, Flask C received 1/25 of 1/25, or 1/650, of the original amount of "bios," and Flask J received 1/5, 667,253,614,400; *i.e.*, less than one-five trillionth of the "bios" in the one loop of yeast cells used to seed Flask A. If, as Amand (12) supposes, the original amount of "bios" decreases very rapidly, it must appear that yeast may grow without "bios" or water-soluble B, or it must be able to synthesize its own supply.

The fermentative power of the yeast used in these experiments was determined for cells taken from the fifteenth seeding. 0.5 cc. of yeast suspension was used for each test. The examination was carried out according to Durham's fermentation test with glucose solution.

	Gas. *inches*
Yeast XII after 3 days	3½
" K " 3 "	3¾
" F " 3 "	3½
Control " 3 "	0

There can be no question about the remarkably stimulating effect of adding various substances such as amino-acids and other extractives to such purified nutrient solutions as were used in this work. Where extracts of natural foods are employed, using yeast as a test organism with a view to determining by the extent of the proliferation of the cells the content of antineuritic substance in such extracts, there is always added with the antineuritic substance so many others having a favorable influence on the growth of yeast that it seems unlikely that the test as carried out by Williams and Bachmann contains any element of specificity.

SUMMARY.

The results of our efforts to cultivate yeast in nutrient solutions containing no possible source of the antineuritic factor have been of such a nature as to make us seriously question whether yeast is dependent on a supply of the antineuritic principle for its continued multiplication. Successive seedings with very few cells have been carried sufficiently far to reduce the possible content of antineuritic substance ("bios," "water-soluble B") to vanishingly small amounts.

We have grown quantities of yeast in this purified nutrient solution sufficient to furnish from 2 to 5 gm. of dry substance. It would seem that but one of two conclusions is admissible; *i.e.*, either yeast must grow without "bios" or it must synthesize the substance to meet its own needs (as was believed by Henry (13)). Feeding experiments with yeast grown under the conditions described in this paper are contemplated and should yield results of unusual interest.

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THE DETERMINATION OF SODIUM IN BLOOD.*

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A knowledge of the amounts of sodium salts in tissues and biological fluids is of considerable importance in reaching an understanding of the physiological rôle of inorganic substances. However, an adequate study of the variations in the amount of sodium in blood, for instance, under different physiological and pathological conditions would be difficult if not impossible by methods[1] now in use. The quantitative determination of sodium by existing methods requires the ashing of the material, the removal of sulfates, phosphates, iron, calcium, and magnesium, and the estimation of potassium in the weighed alkali chlorides. Such a long and tedious procedure seriously limits a study of the metabolism of sodium. Another limiting factor is the amount of material necessary for an analysis, at least 15 cc. of blood being required for the usual gravimetric method.

These considerations prompted the search for a shorter method for the determination of sodium in smaller amounts of material. It seemed essential that some insoluble compound of sodium be found which would permit its quantitative precipitation. The most promising of the very few sodium compounds having small solubility appeared to be the complex sodium cesium bismuth nitrite described by Ball (2) and used by him for sodium determinations. A method based upon the precipitation of this

* A preliminary report of this method was made at the meeting of the American Society of Biological Chemists at Cincinnati, December, 1919.

[1] Kramer (1) has recently described a method for the determination of sodium based upon the insolubility of the pyroantimonate. We have had no experience with this method, but Kramer's data indicate that it is a satisfactory procedure for the estimation of sodium in small samples of tissue.

compound has proved to be readily applicable to blood and urine and with some modification should be equally serviceable for any tissue. The results are believed to be almost as accurate as those obtained by the older procedures while the amount of material and time required are much less. As small an amount of sodium as 0.01 mg. yields a precipitate in a final volume of 2 cc. None of the ions commonly occurring with sodium interferes with the precipitation.

Certainly this method has great promise. The chief objections to it as described by Ball are the formation of a scum of bismuth subnitrate during the precipitation of the complex sodium salt, and the solubility of the latter in all solvents of the precipitating reagent. After many experiments we feel that we have overcome these difficulties.

The formation of the scum is probably due to a loss of nitrous acid and a consequent decrease of acidity of the solution. We find that if the reagent is added to the cold solution of the sodium salt and the flask immediately put in a cold room (1°C.), a scum rarely forms within 24 hours. At this temperature the precipitation of the complex nitrite is more rapid and is generally complete in 18 hours.

All the solutions used by Ball for washing the precipitate in order to remove the mother liquor dissolve so much precipitate that serious errors are introduced. He attempted to apply a solubility correction which would amount to 10 or 15 per cent of the precipitate in some of our determinations. However, none of the solutions that we have tried was so efficient as the 50 per cent acetone in removal of the excess reagent. Consequently we have retained it but have reduced the solubility of the precipitate in it to almost zero. The 50 per cent acetone is saturated with solid sodium cesium bismuth nitrite at 1°C. The excess salt is filtered off at this temperature and the solution used for washing. Its temperature probably does not rise more than 2 or 3° before the washing is complete.

The method as published by Ball was gravimetric. We have been able to show that the nitrite is susceptible of both volumetric[2] and colorimetric estimation. The nitrite may be oxidized

[2] Faber and Stoddard (3) have used the sodium method described by Ball for the analysis of potassium nitrate. They mention the titration of

to nitrate on titration with standard $KMnO_4$. 0.1 N permanganate is suitable since the precipitate from 1 mg. of sodium requires 4.35 cc. for oxidation of nitrite to nitrate.

$$9\ CsNO_2 \cdot 6\ NaNO_2 \cdot 5\ Bi(NO_2)_3 = 30\ NO_2$$
$$30\ NO_2 + 15\ O_2 = 30\ NO_3$$

1 gram-molecule (3,753.6 gm.) of the complex nitrite requires 15 gram-molecules or 480 gm. of oxygen for oxidation.

$$\frac{9\ CsNO_2 \cdot 6\ NaNO_2 \cdot 5\ Bi(NO_2)_3}{30 \times 16} = \frac{3753.6}{480} = 7.82$$

i.e., 1 mg. of O will be required for 7.82 mg. of the precipitate.
1 mg. of sodium produces 27.2 mg. of complex nitrite. Consequently $\frac{27.2}{7.82} = 3.48$ mg. of O to oxidize precipitate from 1 mg. of sodium.

$$\frac{3.48}{0.8} = 4.35 \text{ cc. } 0.1\ N\ KMnO_4$$

The colorimetric estimation is based on the coupling reaction of Griess (4) using napthylamine and sulfanilic acid. Advantage is taken of the fact that bismuth salts are soluble in alkaline tartrate solutions. The precipitate is dissolved in alkaline potassium tartrate, made up to a definite volume, and a suitable sample taken. The standard is 0.01 mg. of nitrite nitrogen. The colors are developed and read with a Duboscq colorimeter.

Reagents.

1. Bismuth Cesium Nitrite Solution.—Although we have attempted to improve Ball's reagent in several ways, we have been unsuccessful. It is much less stable at room temperature than at 1°C. If kept under an inert gas at 1°C. it is suitable for quantitative work for several weeks.

the precipitate with permanganate but give no data from which one may judge of its accuracy.

The authors are indebted to Mr. Faber and Mr. Stoddard for suggesting the possibilities of Ball's method for the determination of small amounts of sodium.

30 gm. of sodium-free potassium nitrite[3] are dissolved in about 60 cc. of pure water. A solution containing 3 gm. of bismuth nitrate is added. (We keep on hand a 60 per cent solution of the crystallized salt in 2 N HNO_3.) If a precipitate forms (due to excessive alkalinity of the KNO_2), dilute nitric acid is added carefully until it redissolves. A strong solution containing 1.6 gm. of $CsNO_3$ and 1 cc. of 2 N HNO_3 is added. The solution is diluted to 100 cc. and dilute nitric acid is used to remove any turbidity which may form. At this stage the reagent should be a clear orange-yellow. If sodium salts were present in any of the chemicals as impurity, the insoluble precipitate which has formed at the end of 24 hours is filtered off. The reagent is kept under illuminating gas in the cold room.

2. Acetone.—Redistilled and kept ready for use at 1°C.

3.—A 50 per cent solution of acetone saturated at 1°C. with sodium cesium bismuth nitrite.

For Volumetric Procedure.—

1. Permanganate, 0.1 N or 0.05 N.
2. Oxalic acid, 0.1 N or 0.05 N.
3. H_2SO_4, concentrated acid diluted with equal volume of water.

For Colorimetric Procedure.—

1. Alkaline tartrate. Equal volumes of KOH (10 per cent) and tartaric acid (10 per cent) are mixed.
2. Sulfanilic acid, 0.8 per cent in 5 N acetic acid.
3. α-Naphthylamine, 0.5 per cent in 5 N acetic acid.

[3] Pure potassium nitrite has been a source of considerable difficulty which we have finally overcome. The potassium salts on the market generally contain large quantities of sodium. Since the nitrite cannot be purified by recrystallization, our only recourse was to make it. We have examined various samples of carbonate and have found that both Merck's Blue Label and Eastman's are substantially free from sodium. As an emergency procedure sodium-free potassium carbonate may be made by recrystallization of the oxalate. It is dried and ignited in a platinum dish.

The pure nitrite is made by passing nitrous fumes into a 25 per cent solution of sodium-free potassium carbonate. Nitric acid (sp. gr. 1.2) is dropped from a separatory funnel into a flask containing arsenious oxide. A delivery tube carries the fumes into the carbonate. The reaction is complete when the solution in the receiving flask gives off many fine bubbles of carbon dioxide on shaking. We generally run nitrite determinations at intervals to ascertain whether the reaction is running properly.

4. Nitrite standard. Made by recrystallizing $AgNO_2$ from hot water until free from nitrate. Add NaCl equivalent to the $AgNO_2$ and filter off silver chloride. Determine nitrite nitrogen by Devarda's (5) method and dilute so that 5 cc. = 0.01 mg. N.

Preparation of the Material for Analysis.

When we started our analyses of blood and urine we thought that it would be necessary to remove the organic material by ashing. We have used both the wet and dry methods. In our hands the former has been the more successful. As many of our data were obtained on blood ashed with sulfuric and nitric acids, this procedure is given below in detail.

1 cc. of whole blood, plasma, or urine is transferred to a pointed Pyrex tube.[4] A few drops of H_2SO_4 (concentrated) and 5 cc. of HNO_3 (concentrated) are added. A low flame which keeps the liquid boiling gently is used. The digestion is continued in the usual manner until the liquid is colorless. Urine is completely oxidized in about 8 minutes but the blood generally takes ¾ hour.

As both iron salts and any appreciable amount of phosphates interfere with this method of determination of sodium, they must be removed from the blood digest. None of the samples of urine or plasma analyzed contained sufficient quantities to vitiate the analysis.

The digest of the whole blood is quantitatively transferred to a 25 cc. volumetric flask with about 20 cc. of water. 1 drop of methyl orange and 5 to 6 drops of 4 per cent bismuth nitrate are added. A strong solution of potassium carbonate (free from sodium) is added dropwise with shaking until the indicator changes color. The flask is made up to volume, mixed, and the solution transferred to a centrifuge tube. Centrifuging at moderate speed throws down the insoluble phosphates and iron salts.

[4] These tubes are reclaimed from non-protein nitrogen determinations. After a tube has been rendered unserviceable by the phosphoric acid it is heated in an oxygen-gas flame and drawn out to a point. The tubes with small tips and thin walls stand heating best. The pointed tip provides a constant stream of bubbles which promote even boiling. No boiling stones are necessary.

20 cc. of the supernatant liquid are pipetted into a 50 cc. Erlenmeyer flask. This solution is evaporated[5] on the hot plate to 2 to 3 cc. and rendered just acid with HNO_3. An excess of 0.5 cc. of 2 N HNO_3 is added and the precipitation carried out as described.

After carefully checking our method on the ashed blood, we found that a great deal of time and labor could be saved by deproteinization with trichloroacetic acid. Our data (Table I) indicate that a dilution of 1:5 or 1:10 is perfectly satisfactory.

The procedure is as follows. 5 cc. of whole blood or plasma are transferred to a 50 cc. flask containing 35 cc. of water, and 5 cc. of trichloroacetic acid (20 per cent) are added. The contents of the flask are diluted to the mark, mixed, allowed to stand about 30 minutes, and filtered through a dry paper. 10 cc. of filtrate (equivalent to 1 cc. of blood) are pipetted into a 50 cc. Erlenmeyer flask and 1 drop of concentrated nitric acid is added. The flask, closed with a trap, is heated on a piece of asbestos on a hot plate until brown fumes from the acid are evolved. It is removed, cooled, and the trap washed off with a few drops of water. Precipitation is then carried out as described below.

Precipitation.—The solution is cooled to 10–12°C. and 3 cc. of reagent are added for each milligram of sodium expected. The flask is stoppered with a two-hole rubber stopper bearing two short glass tubes bent at a right angle. One is fitted with a short rubber tube with a glass plug, the other with a Bunsen valve and plug. Illuminating gas freed from H_2S is passed into the flask for a few seconds and the plugs are replaced. The flask is put in the cold room at 1°C. A yellow crystalline precipitate begins to form in a few minutes. Precipitation is complete in 24 hours, whereas at room temperature 48 hours are required. A scum is much more likely to form before the precipitation is complete at the higher temperature.

The precipitate is rapidly filtered on a Gooch crucible which has previously been dried and weighed. Washing with the ice-cold 50 per cent acetone which is saturated with sodium

[5] We advise the use of a trap in the mouth of the flask to prevent loss by bumping. Ours is made by blowing a bulb on the closed end of a small soft glass test-tube. A hole is then blown in the side of the bulb which is then cut off from the tube.

cesium bismuth nitrite is quickly carried out. Speed during the filtration and washing is essential for good results. 10 cc. of the 50 per cent acetone are used; 2 cc. are blown from a miniature wash bottle (made from a 10 cc. graduated cylinder) into the precipitation flask. The suction is stopped and the liquid poured onto the mat. This is repeated four times. 10 cc. of pure acetone are used to complete the transfer of the precipitate to the Gooch. If the volumetric or colorimetric procedure is used, complete transference of the precipitate is not necessary.

The Gooch is dried in an air bath at 100°C. until a constant weight is obtained.

Weight of precipitate × 0.03675 = Weight of sodium in solution

We prefer to avoid the weighing necessary for a gravimetric determination. Incidentally any scum which may have formed will cause the gravimetric result to be too high but will not affect the nitrite estimation. Very frequently potassium nitrate crystallizes at 1°. This will also give erroneous gravimetric results. The precipitate is filtered as described on a Gooch crucible and estimated either volumetrically or colorimetrically.

Volumetric Procedure.—The Gooch crucible and contents are placed in a tall 200 cc. beaker. A large excess of standard permanganate (at least twice the amount necessary for oxidation) and enough water to cover the crucible are added. The precipitate is stirred loose from the crucible and asbestos. 10 cc. of 1:1 sulfuric acid are poured in while the liquid is being stirred. After a few minutes the solution is heated to 75°C., an excess of standard oxalic acid added, and the titration finished with permanganate. A blank must be run on the reagents under similar conditions.

Cc. $KMnO_4$ × normal factor × 8 = Mg. O used
Mg. O × 7.82 = Mg. precipitate
Mg. precipitate × 0.03675 = Mg. sodium

Colorimetric Procedure.—For those who prefer a colorimetric method we have established a suitable technique. The precipitate is completely transferred to a beaker and 10 cc. of the alkaline tartrate are added. Upon warming, the salt rapidly dissolves. The solution is quantitatively transferred to a 100 cc. volumetric

flask, cooled, made up to volume, and mixed. A further dilution is made so that a volume containing approximately 0.01 mg. of N can be taken for colorimetric comparison.

The standard and unknown in 100 cc. volumetric flasks are diluted to about 90 cc. 2 cc. of the sulfanilic acid and napthylamine solutions are added to each. The flasks are made up to volume, mixed, and allowed to stand 20 minutes for the full color development. There is a very wide range of proportionality of color intensity to the amount of nitrite present. The colors are very stable.

The calculation is simple.

$$\frac{20 \times 0.01}{\text{Unknown reading}} = \text{Mg. N in sample used}$$

Suppose the sample was 1 cc. of a dilution of 1:1,000 then

$$\text{Mg. N in sample} \times 1{,}000 = \text{Mg. N in precipitate}$$

$$\frac{9\ CsNO_2 \cdot 6\ NaNO_2 \cdot 5\ Bi(NO_2)_3}{30\ N} = \frac{3753.6}{420.3} = 8.93$$

$$\text{Mg. N} \times 8.93 = \text{Mg. precipitate}$$

$$\text{Mg. precipitate} \times 0.0367 = \text{Mg. sodium in sample}$$

We prefer the volumetric method to either the gravimetric or colorimetric on account of its greater speed and accuracy. Possible contamination of the precipitate with either bismuth subnitrate or potassium nitrate renders the gravimetric values doubtful. The colorimetric procedure is open to the usual errors of such methods. The red color is very bright and comparison is rather difficult.

In Table I the figures given under "Indirect" were obtained by ashing the material in a platinum dish. The ash was taken up in water, acidified with hydrochloric acid, and the sulfates were precipitated. The barium sulfate was filtered off and the filtrate made alkaline with ammonia. After the barium phosphate had precipitated, an excess of ammonium carbonate was added. The precipitate was filtered off and the filtrate evaporated in a platinum dish. The salts were carefully dried and gently ignited. The salts were dissolved in water and tested for complete removal of barium and calcium. Generally a small amount of insoluble

material was present. It was filtered off and the filtrate caught in a small weighed platinum dish. Evaporation to dryness, and ignition were carried out as previously described.

TABLE I.

Sodium in Urine and Blood.

Experiment No.	Source of sample.	Sodium per 100 cc.			Method.
		Indirect.	Direct ash.	Trichloroacetic acid filtrates.	
		mg.	*mg.*	*mg.*	
1	Urine, normal.	414	403		Volumetric.
			407		Gravimetric.
2	" "	351	349		Volumetric.
			341		"
3	" "	351	348		"
			343		"
4	Blood, beef.	284	285		Gravimetric.
			279		"
5	" "	278	268		"
			270		"
6	" "	272	263		"
			265		"
7	" "	266		265	"
8	" "	281		272	Colorimetric.
				275	"
				283	Volumetric.
9	Plasma, "	336	339	342	Colorimetric.
				343	Volumetric.
10	Blood, swine.	221	219		"
			226		"
11	" "	206	204		"
			212*		"
12	" "	217		216	"
13	" "	209	212	213	Colorimetric.
14	Plasma, "	360	358	360	"
				358	"
				352	Volumetric.

* Determination on 0.4 cc. of blood.

The mixed sodium and potassium chlorides were dried to constant weight and the potassium was determined in the usual manner as perchlorate. The sodium perchlorate was dissolved in

97 per cent alcohol containing 0.2 per cent perchloric acid. Consequently the results for potassium are possibly a little low and sodium a little high (6).

Table II illustrates the accuracy to be expected when weighed quantities of sodium cesium bismuth nitrite are titrated with

TABLE II.

Titration of Sodium Cesium Bismuth Nitrite with Permanganate.

Experiment No.	Amount of pure salt.	Amount by titration.	Error.
	mg.	*mg.*	*mg.*
1	62.6	62.0	−0.6
2	55.3	55.8	+0.5
3	89.4	88.4	−1.0
4	51.5	52.1	+0.6
5	54.1	55.1	+1.0
6	118.6	118.0	−0.6

TABLE III.

Determination of Sodium in Pure Sodium Nitrate.

Na taken.	Na found.	Time for precipitation.	Temperature.	Method.
mg.	*mg.*	*hrs.*	*°C.*	
1.0	0.96	44	22	Gravimetric.
1.0	1.01	65	22	Volumetric.
1.0	0.99	69	22	"
2.0	1.62	20	22	Gravimetric.
2.0	2.02	44	22	Volumetric.
2.0	1.97	44	22	"
2.0	2.03	44	22	"
2.0	2.04	44	22	Gravimetric.
2.0	1.85	7	1	Colorimetric.
2.0	1.92	15	1	"
2.0	2.04	24	1	"
3.0	3.06	44	22	Gravimetric.
3.0	2.98	24	1	Volumetric.

permanganate. The volumetric estimation of known amounts of sodium salts also throws light on the reliability of a titration procedure.

Table III gives some values obtained by the gravimetric, volumetric, and colorimetric procedures on pure sodium nitrate.

Our early work in agreement with Ball's demonstrated that at least 44 hours were necessary for the complete precipitation at room temperature. We have found 24 hours to be sufficient at 1°C. 15 hours at this temperature gave low results.

Table I shows a comparison of values obtained by the usual procedure and by our different modifications of the sodium cesium bismuth nitrite method. We think that the results are sufficiently accurate for most work but hope to increase their accuracy by a few refinements on which we are now working.

Attention should be called to the constancy of the amount of sodium in the blood of the same species. In the case of both swine and beef the maximum variation is only about 7 per cent.

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A STUDY OF THE DISTRIBUTION OF IODINE BETWEEN CELLS AND COLLOID IN THE THYROID GLAND.

II. RESULTS OF STUDY OF DOG AND HUMAN THYROID GLANDS.

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In the first paper of this series Tatum[1] described a method whereby thyroid cells may be separated from colloid material and examined chemically. Briefly the method consists in cutting frozen sections of the thyroid gland and floating these sections on Ringer's[2] solution. The colloid material immediately drops out of the acini and is presumably dissolved in the Ringer's solution. The cells may then be separated by centrifugalization, dried, weighed, and analyzed. Comparisons between the iodine content of cells so separated and the iodine content of control pieces of unsectioned whole gland may indicate the distribution of iodine between cells and colloid under different functional conditions.

In this paper I wish to report the results of a study, suggested by Dr. A. L. Tatum, of the distribution of iodine in the thyroid glands of normal and iodine-fed dogs as well as in human glands obtained from individuals subjected to operation for toxic goiter. The method earlier described by Kendall[3] was used in making the final iodine determinations.

Incidental to the determination of the iodine distribution in dog and human thyroid glands, some control experiments were performed relative to the alterability of the intracellular iodine concentration during the process of cutting. It may be argued that a portion of the iodine-containing compound diffuses from

[1] Tatum, A. L., *J. Biol. Chem.*, 1920, xlii, 47.
[2] Campbell, J. A., *Quart. J. Exp. Physiol.*, 1911, iv, 1, Formula "A."
[3] Kendall, E. C., *J. Biol. Chem.*, 1914, xix, 251.

cells as they lie suspended in Ringer's solution during the separation of the colloid material. If it is assumed that such an outward diffusion takes place, it seems reasonable to expect that the iodine compound should diffuse back into the cells if we should increase the concentration of that iodine compound in the Ringer's solution in which the cells are suspended. Several experiments like those given in Table I were undertaken to determine whether or not the iodine content of the cells could thus be increased.

TABLE I.

Effect of Floating Cells on Pure Ringer's Solution and on Ringer's Solution on Which Cells of an Iodine-Rich Gland Had Been Floated.

Animal No.	Weight of whole gland used.	Iodine in whole gland.	Weight of cell mass used.	Iodine in cell mass.	Ratio of percentage of iodine in cells to percentage of iodine in whole gland.	Remarks.
	mg.	*per cent*	*mg.*	*per cent*		
14	146.0	0.187	40.5	0.017	0.091	Cells floated on pure Ringer's solution.
			53.0	0.017	0.091	Cells floated on Ringer's solution on which previously cells of iodine-rich gland had been floated.
20	287.2	0.030	161.5	0.005	0.167	Cells floated on pure Ringer's solution.
			151.0	0.006	0.20	Cells floated on Ringer's solution on which previously cells of iodine-rich gland had been floated.

It may be seen from the data given in Table I that no increase in the iodine content of cells could be brought about by increasing the concentration of the characteristic iodine compound in the Ringer's solution.

Again the iodine concentration of the cell mass does not seem to be in the least altered whether or not the freshly centrifugalized cell mass is washed several times with iodine-free Ringer's solution.

Moreover, in the attempt to find a suspending medium more nearly related physicochemically with thyroid cells than is Ringer's solution, I have used fresh dog serum and have not found the iodine content of the cells measurably different from that of control cells suspended in Ringer's solution.

Finally autolysis does not seem to be much of a factor in the loss—if any occurs—of iodine compounds from cells as they lie in contact with Ringer's solution. Throughout the process of cutting and separating the cells, the reaction of the suspending medium should remain weakly alkaline—a reaction which has been shown to be unfavorable to autolysis.[4] Also in experiments to be reported later in which the same technique was used under slightly different conditions, every effort was made to eliminate autolysis by cutting successively small portions of the gland and floating the cells on Ringer's solution cooled by ice. The cells from the small portions cut successively were at once centrifugalized and dried. No change could be noticed in the iodine distribution.

Results of Study of Dog Glands Taken at Random.

TABLE II.

Quantitative Determination of Iodine in Whole Gland and in Cells Free from Colloid Material of Thyroid Glands of Normal Dogs.

Animal No.	Weight of whole gland used.	Iodine in whole gland.	Weight of cell mass used.	Iodine in cell mass.	Ratio of percentage of iodine in cells to percentage of iodine in whole gland.	Morphology.	
						Cells.	Colloid.
	mg.	*per cent*	*mg.*	*per cent*			
5	550.0	0.25	154.0	0.031	0.124	Flat.	Fair.
6	388.0	0.175	265.0	0.027	0.154	"	Rich.
10	480.0	0.017	342.0	0.003	0.176	Cuboidal.	Poor.
11	505.5	0.032	197.5	0.005	0.156	"	"
12*	278.7	0.006	286.5				
13	689.0	0.234	154.0	0.047	0.201	Flat.	Fair.
14	146.0	0.187	40.5	0.017	0.091	"	"
19	474.5	0.024	116.0			"	Poor.
20*	287.2	0.030	161.5	0.005	0.167		
21*	604.7	0.038	189.0	0.006	0.158		
23	458.2	0.003	390.4			Cuboidal.	Poor.
24	318.7	0.021	295.4			"	"
31	513.8	0.055	205.6	0.008	0.145	"	"
32*	235.2	0.179	103.9	0.037	0.207		
33	289.3	0.011	538.3	0.002	0.182	Flat.	Poor.

* No histological examination.

[4] Bradley, H. C., *J. Biol. Chem.*, 1915, xxii, 113. Bradley, H. C., and Taylor, J., *J. Biol. Chem.*, 1916, xxv, 261.

From the results given in Table II one sees that the ratio of iodine concentration in cells to iodine concentration in whole gland (and hence the ratio of iodine concentration in cells to colloid-iodine concentration) has a quite constant value. In some glands (Nos. 12, 19, 23, and 24), to be sure, the iodine content of the cell mass analyzed was so low that no ratio value could be obtained. But in general, despite great variations in the iodine content and morphology of the glands analyzed, the ratio values change relatively little. These findings in the dog's thyroid gland are similar to those of Tatum[1] in the beef, pig, and sheep thyroid glands. A comparison of the ratio values in different animals is given below.

Effect of Feeding Iodine.

It was thought that perhaps ratio changes could be induced by the administration of iodine or iodine compounds. Capsules containing 1 gm. of potassium iodide or 2 drops of tincture of iodine in starch were fed over varying periods of time. No more than one capsule was given in 24 hours. The results of the examination of the glands of animals so treated are given in Table III.

The ratio obtained from analyzing the cells and whole gland of the thyroid glands of animals which had received varying amounts of iodine or iodine compounds over periods of no less than 3 days is practically the same as that of the animals which had received no iodine. One gland (that of No. 16) gave an unusually high value which I cannot explain. In view of the variations in morphology and iodine content of the glands of the normal series together with the quite constant ratio exhibited by that series, it is not surprising that the ratio still remains constant despite the feeding and consequent absorption of iodine. From a consideration of the glands examined it appears that the ratio is not altered by the feeding of iodine as potassium iodide or free iodine over periods of time ranging from 3 days to 3 weeks; and yet the total iodine content of these glands undoubtedly is greatly increased during that same period.

TABLE III.

Quantitative Determination of Iodine in Whole Gland and in Cells Free from Colloid Material of Thyroid Glands of Iodine-Fed Dogs.

Animal No.	Form of iodine.	Period of feeding.			Weight of whole gland used.	Iodine in whole gland.	Weight of cell mass used.	Iodine in cell mass.	Ratio of percentage of iodine in cells to percentage of iodine in whole gland.
		Daily.	On alternate days.*	Total.					
		days	*days*	*days*	*mg.*	*per cent*	*mg.*	*per cent*	
2	KI	14	0	14	98.2	0.31	77.7	0.045	0.145
7	KI	14	7	21	147.0	0.12	113.0	0.031	0.258
8	KI	14	7	21	124.5	0.87	125.0	0.080	0.092
9	KI	14	2	16	353.5	0.251	116.0	0.040	0.159
16	KI	12	0	12	651.4	0.319	316.1	0.117	0.367
17	KI	12	0	24	646.5	0.617	302.3	0.083	0.134
	Tincture of I.	12	0						
27	" " I.	3	0	3	556.1	0.302	332.8	0.046	0.152
29	" " I.	3	0	3	195.2	0.314	158.6	0.039	0.124
30	" " I.	3	0	3	163.3	0.357	127.5	0.041	0.115

* After feeding daily.

Human Glands.

The distribution ratio of iodine between cells and colloid material was determined in thirteen human glands obtained from operative cases.[5] In Table IV are given the results of the analyses of the human glands together with the clinical diagnosis made in connection with ten of the cases.

It will be seen that most of the thyroid glands reported were clinically diagnosed as toxic goiters. Considerable variations in the ratio value occur and do not appear to be related either to the iodine content of the gland or to its morphology. The ratio value of No. 22 is inexplicably high. In the human gland series, however, as in other gland series previously reported, the ratio variations are of a much smaller magnitude than the variations in total iodine content. So here too the ratio is fairly constant despite variations in morphology and iodine content.

[5] Through the courtesy of Dr. C. B. Davis and Dr. A. D. Bevan of the Presbyterian Hospital, Chicago, and of Dr. A. J. Ochsner of Augustana Hospital, Chicago.

TABLE IV.

Quantitative Determination of Iodine in Whole Gland and in Cells Free from Colloid Material of Human Thyroid Glands.

Series No.	Weight of whole gland used.	Iodine in whole gland.	Weight of cell mass used.	Iodine in cell mass.	Ratio of percentage of iodine in cells to percentage of iodine in whole gland.	Morphology.		Diagnosis.
						Cells.	Colloid.	
	mg.	*per cent*	*mg.*	*per cent*				
22	284.7	0.081	260.8	0.049	0.605	Cuboidal.	Poor.	Colloid cystic goiter with toxic symptoms.
25*	600.2	0.274	308.6	0.105	0.383	Flat.	Rich.	Mild exophthalmic goiter.
	411.0	0.276	240.7	0.098	0.355			
35	671.8	0.286	179.2	0.051	0.178	Flat.	Rich.	Toxic thyroid.
36	439.7	0.158	219.9	0.020	0.127	Cuboidal.	Fair.	" goiter.
37†	667.5	0.092	258.4	0.027	0.293			Colloid goiter.
38	578.6	0.079	191.7	Trace.		Cuboidal.	Poor.	" " following parenchymatous hyperplasia of exophthalmic goiter.
39†	516.0	0.279	297.6	0.095	0.341			
40	469.2	0.345	225.5	0.066	0.191	Flat.	Rich.	Exophthalmic goiter.
41	559.2	0.280	187.0	0.067	0.239	"	"	
48*	549.9	0.043	312.4	0.005	0.116	Cuboidal.	Poor.	
	607.3	0.044	311.3	0.005	0.114			
49*	590.7	0.137	256.0	0.026	0.189	Cuboidal.	Fair.	Exophthalmic goiter.
			204.5	0.027	0.197			
51	731.4	0.283	294.4	0.072	0.254	Flat.	Rich.	" "
60	370.0	0.152	228.0	0.015	0.099	"	"	Cystic goiter with toxic symptoms.

* Duplicate determinations made.

† No histological examination

Comparison of Ratio Value in Different Animals.

A comparison of the ratio values of the thyroid glands of the different animals so far examined is given in Table V. The few abnormally high and unexplained ratio values are not included in the table.

From what data are available there appear to be some differences in the thyroid glands of different animals in the numerical value of the ratio of the percentage of iodine in cells to that in whole gland. The ratio value of iodine distribution for dog thyroid glands seems to be consistently lower and more constant than that for the thyroid glands of the other animals studied.

TABLE V.

A Comparison of the Value of the Ratio of the Percentage of Iodine in Cells Free from Colloid Material to the Percentage of Iodine in Whole Gland in Different Animals.

Animal.	Extremes of iodine content of whole gland.	Ratio values.		
		Extremes.	Mean.	Average.
	per cent			
Beef*	0.023–0.468	0.21 –0.48	0.35	0.36
Dog	0.011–0.870	0.091–0.258	0.175	0.154
Man	0.043–0.345	0.099–0.384	0.242	0.22
Pig*†	0.377–0.810	0.20 –0.34	0.27	0.27
Sheep*	0.089–0.442	0.23 –0.41	0.32	0.33

* Tatum.[1]

† Only two glands analyzed.

SUMMARY.

1. The method described by Tatum[1] was used to determine the ratio of the percentage of iodine in cells to the percentage of iodine in whole gland in the thyroid glands of normal and iodine-fed dogs as well as in human thyroid glands obtained from operative cases.

2. Evidence is presented indicating that the concentration of intracellular iodine is independent of the suspending medium, whether that is pure Ringer's solution, Ringer's solution containing iodine-rich colloid material, or homologous blood serum.

3. The ratio value was found to be relatively constant despite great variations in the morphology and iodine content of the glands examined. The ratio value for the dog's thyroid gland seems quite constant and is much lower than that of the beef or sheep.

STUDIES ON EXPERIMENTAL RICKETS.

I. THE PRODUCTION OF RACHITIS AND SIMILAR DISEASES IN THE RAT BY DEFICIENT DIETS.

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Plates 2 and 3.

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Students of the etiology of rickets have advanced the most diverse views to account for its occurrence. Heredity, dietetic factors, faulty hygienic conditions, infections of a microbial or other nature, and disturbances of the endocrine glands have all been incriminated as the causative agents.

In the conduct of nutritional experiments on the rat which have been carried on by the members of the Department of Chemical Hygiene of the Johns Hopkins University for more than a decade, it has been the custom to autopsy all animals which have died from any cause. In the course of these routine autopsies pathological conditions of the skeleton, *viz.* enlargement of the epiphyseodiaphyseal junction, fractures of the ribs, enlargement of the costochondral junctions and vertebral ends of the ribs, abnormal spinal curvatures, etc., have been observed repeatedly and have been regarded as evidences of rickets. Other problems prevented for a time any effort at intensive study of the nature of the factor or factors which operate to produce these conditions. There was never any room for doubt that the diet was the sole determining factor in the etiology of the lesions in question, for the entire colony of approximately 2,000 animals lived under identical conditions except for those related to the composition and source of their food.

Although the members of the Department of Chemical Hygiene were of the opinion that the skeletal malformations found in their rats were the result of rickets, it was not possible for them alone to arrive at definite conclusions owing to a lack of the necessary pathological experience. It so happened that the Department of Pediatrics had been engaged for a number of years in the study of rickets from the chemical and pathological standpoints and had already conducted experiments with a view to the production of rickets in animals. It seemed advisable, therefore, for the two departments to unite in a cooperative study concerning the origin and nature of the lesions which had been produced in the rat as the result of the varied and presumably insufficient diets. In this undertaking the Department of Chemical Hygiene is responsible for the planning of the diets which have produced the disease, and their administration, the care of the animals, and the interpretation of the nature of the faults in the food mixtures themselves. The Department of Pediatrics has assumed the responsibility for the determination of the exact nature of the pathological processes and has undertaken the interpretation of them in the light of human osteopathology and in so far as it is possible the correlation of the osteo- and organopathology to the dietary defects. Each department has utilized the information gained from the other so as to plan most effectually for future experiments.

The present paper, the first of a series, has for its purpose the presentation of certain faulty diets which when fed to the young rat produce several disturbances in the growth of the skeleton which have in common fractures, enlargement and distortion of the costochondral junctions, vertebral deformity, the overproduction of osteoid tissue, and irregularities in the calcification of the intercellular substance of the proliferative cartilage or absence of lime salt deposits from the matrix of this tissue. Administration of some of these diets to young rats results in the development of pathological changes in the osseous system which are identical with those found in the bones of rachitic children. Others of the dietary formulas used caused changes in the bony and cartilaginous skeleton which bear a close resemblance to those found in human rickets but can only be said to be similar and not identical with this condition. One of these diets

causes in the second generation of rats maintained upon it an extreme degree of osteoporosis with multiple fractures but with relatively slight irregularity in the calcification of the cartilage.

The study which we have undertaken is a most comprehensive one. It has already involved the feeding of several basal rations of a faulty nature supplemented by the addition of single and multiple purified food constituents. This plan originated in the Laboratory of Chemical Hygiene and has been employed for some years in that laboratory as an effective biological method for the analysis of the nature of the deficiencies of the various natural foodstuffs. More than 300 modifications of diet have been effected in producing the rachitic and the related pathological conditions in the skeleton of the rat, and in demonstrating the nature of the faults in the food mixtures. The work of making histological examinations of the rats representative of all the experimental groups has been most time-consuming and is as yet far from being completed.

The present paper is a preliminary communication. We reserve for later papers, therefore, descriptions of the diets and growth curves and the exact pathological conditions produced in the skeleton by each of the diets in question.

In order to appreciate the view we have come to hold relative to the etiology of rickets, the following diets are described and the nature of the defects is pointed out.

No. 2249.

	per cent
Whole wheat kernel	30.0
" maize "	20.0
Polished rice	12.0
Rolled oats	11.5
Peas	12.0
Navy beans	12.0
$NaHCO_3$	1.5
NaCl	1.0

This diet is faulty in that it contains decidedly less than the optimum of both the dietary factors, fat-soluble A and calcium. It is by no means free from either of these essentials. The quality of the protein while not of the best cannot be a factor of importance in inducing malnutrition on this diet.

No. 2581.

	per cent
Beef liver (steamed and dried)	20.0
Casein	10.0
NaCl	1.0
KCl	1.0
Dextrin	65.0
Butter fat	3.0

This diet contains an abundance of protein of good quality and all the inorganic elements necessary for the normal nutrition of the rat except calcium, which is present in very small amount. It is rich in fat-soluble A and contains a fairly liberal amount of water-soluble B. This diet contains a considerable excess of potential acidity. Low calcium and acidity (after catabolism) are the only known faults in this food mixture.

Nos. 2661 and 2677.

	per cent
Rolled oats	40.0
Flaxseed oil meal	8.3
NaCl	1.0
$CaCO_3$	1.5
Dextrin	49.2

This diet is satisfactory for fairly good growth in the rat when supplemented with 5 per cent of butter fat. It has but one serious fault therefore; *viz.*, lack of fat-soluble A. Its proteins are not of a very high biological value but the two seeds when supplemented as they are in this diet with NaCl and $CaCO_3$ are capable of supporting good growth.

No. 2638.

	per cent
Whole wheat kernel	30.0
" maize "	30.0
Polished rice	10.0
Rolled oats	10.0
Peas	10.0
Navy beans	10.0

This diet of cereals and legume seeds is poor in Ca, Na, and Cl ions and also poor in fat-soluble A. Its ash is somewhat acid.

No. 2733.

	per cent
Whole wheat kernel	30.0
" maize "	19.5
Polished rice	9.5
Rolled oats	8.5
Peas	8.5
Navy beans	8.5
Round steak (beef)	10.0
NaCl	1.0
$NaHCO_3$	1.5
Cod liver oil	3.0

Ration 2733, which is essentially the same as No. 2810 but with 1.5 per cent of sodium bicarbonate in place of the dextrin and 3.0 per cent of cod liver oil in place of the butter fat, enables the animals to develop in what appears from their growth curves and external appearance to be a fairly normal manner. The animals so produced have very poor skeletons, however, and the females frequently suffer complete breakdown and death when they attempt to nurse the young.

No. 2810.

	per cent
Whole wheat kernel	30.0
" maize "	14.5
Polished rice	9.5
Rolled oats	9.5
Peas	9.5
Navy beans	9.5
Round steak (beef)	10.0
NaCl	1.0
Dextrin	1.5
Butter fat	5.0

This diet of cereal grains, legume seeds, steak, sodium chloride, and butter fat is satisfactorily constituted for the nutrition of the rat except for its low calcium content. When supplemented with 1.5 per cent of calcium carbonate, growth is normal and health is maintained over a long period of adult life. Successful nutrition of five successive generations of a family of rats has been secured with this food. Without the calcium addition, notwithstanding a high content of butter fat, normal calcification in the bones does not take place.

No. 2811.

	per cent
Bolted flour	30.0
Corn-meal	20.0
Rice	12.0
Rolled oats	11.5
Peas	12.0
Navy beans	12.0
$NaHCO_3$	1.5
Dextrin	1.0

Ration 2811 contains three degerminated products, wheat flour, corn-meal, and polished rice. These are degerminated and decorticated products of the endosperm. The oats, peas, and beans represent the entire seeds. The diet is too low in calcium, fat-soluble A, and protein, and failure of nutrition on this diet is due to these factors which are enumerated in the order of their importance.

No. 2815.

	per cent
Rolled oats	40.0
Gelatin	10.0
Salts (No. 185)	3.7
Dextrin	46.3

Ration 2815 is satisfactory except in that it is very poor in fat-soluble A. The protein is of good quality and the salt mixture is of such a nature as to supplement the deficiencies in the kernel.

No. 2869.

	per cent
Whole wheat kernel	28.0
" maize "	20.0
Polished rice	9.5
Rolled oats	9.5
Peas	9.5
Navy beans	9.5
Round steak	10.0
NaCl	1.0
$CaCO_3$	3.0

This diet is too low in fat-soluble A and too high in calcium. When fed with 2 per cent of cod liver oil the above diet induces normal development of young rats.

No. 2883.

	per cent
Liver	20.0
Casein	10.0
KCl	1.0
NaCl	1.0
Dextrin	66.0
Cod liver oil	2.0

Ration 2883 is satisfactorily constituted except for an extremely low calcium content. Liver is rich in fat-soluble A but in addition the diet contains 2 per cent of cod liver oil which is extremely rich in this factor.

No. 2911.

	per cent
Wheat	30.0
Maize	20.0
Rice	11.0
Rolled oats	11.5
Peas	11.0
Navy beans	11.0
NaCl	1.0
$NaHCO_3$	1.5
Butter fat	3.0

Ration 2911 consists of cereals and legume seeds supplemented with sodium chloride, sodium bicarbonate, and butter fat. In all cases the entire seed was used except rice, which was polished. The limiting factor in this diet for the nutrition of the rat during growth is the calcium content, which is too low. This food mixture with 1.5 per cent of calcium carbonate added is a fairly satisfactory diet for the nutrition of the rat throughout life. The protein is not of the highest quality but is sufficiently good to make possible normal development. The ash of any seed is acid and when seeds are metabolized there results an excess of acid ions in the body. Sodium bicarbonate is added to neutralize this acidity.[1]

Each of the diets described produces in young rats disturbances in the growth and formation of the skeleton. Each of these

[1] In all cases our rations were fed finely ground and so mixed that the individual ingredients could not be picked out and eaten separately by the experimental animal.

diets, when fed supplemented with purified food additions to make good the deficiencies noted in the descriptions of each, is capable of inducing approximately normal nutrition and normal skeletal growth. With these factors corrected there is in no instance any evidence from the general appearance of the animals of the deformities so characteristic of those restricted to the formulas as given in this paper.

The present discussion, as we have said above, is of necessity a preliminary communication and we are not now willing to hazard any statements in regard to the factors operating to produce rickets in the child or the experimental animal. It might seem from a perusal of the diets here discussed (representative of more than 300 formulas) that the cause of these diseases might lie in a deficiency of fat-soluble A or calcium in the food, or a disturbance in the metabolism of these factors. Many years experience with feeding experiments, however, have demonstrated to us how dangerous it is to draw conclusions from apparently obvious experimental data. Any suggestions regarding the absence of a specific antirachitic substance or deficiency of either fat-soluble A and calcium as the primary agent in the production of rickets would be ill considered and might be far from the truth. At present it is only possible to say that the etiological factor is to be found in an improper dietetic regimen. The large variety of dietary formulas the administration of which results in rickets and kindred affection gives abundant evidence of the complex nature of the causes operating in the production of the disease, and we shall attempt the presentation of their analysis in later papers.

EXPLANATION OF PLATES.

h, costochondral groove; *cc*, costochondral junctions; *cv*, costovertebral junctions; *f*, fractures; *d*, shaft; *met*, metaphysis; *os*, calcified bone; *o*, osteoid tissue; *c*, epiphyseal cartilage; *m*, bone marrow; *dia*, diaphysis; *ep*, epiphysis.

PLATE 2.

FIG. 1. Rachitic rat (R) contrasted with a normal animal (N) of the same age and sex (210 and 204 days respectively). The rachitic animal weighed 100 gm., the normal animal 295 gm.

FIG. 2. Pleural surface of the thorax from a case of severe experimental rickets showing the extreme enlargement at the costochondral junctions.

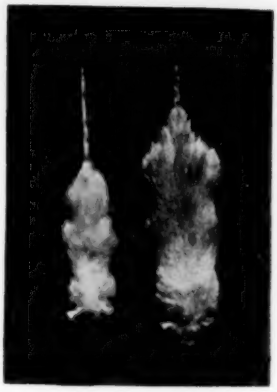
FIG. 3. An extreme case of experimental rickets. Cross-section of thorax at the level of the fifth costochondral junction. The cavity of the chest is almost completely divided into two parts by the bowed ribs and the enlargement of the costochondral junctions. Nearly the entire lungs were forced into the posterior chamber, the heart occupying the anterior chamber of the thorax. This section shows how much more marked the costochondral enlargement is on the pleural than on the external surface of the thoracic wall.

FIG. 4. Rachitic kyphoscoliosis. This picture shows also the enlargement of the ribs at the costovertebral junctions.

FIG. 5. Pleural surface of thorax from a case of experimental rickets to show multiple spontaneous fractures (healing) along the ribs.

PLATE 3.

FIG. 6. Photomicrograph of a section through the distal epiphysis and shaft of femur of a rat with experimental rachitis. The picture shows the overgrowth and irregular invasion of the epiphyseal cartilage, the presence of a metaphysis, and the overproduction of osteoid tissue. × 250.



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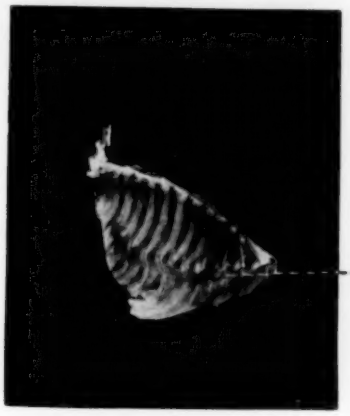

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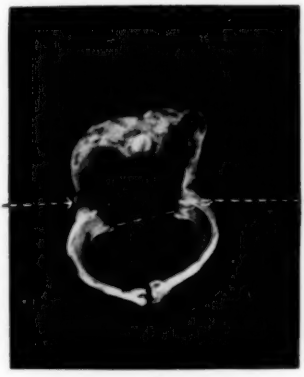

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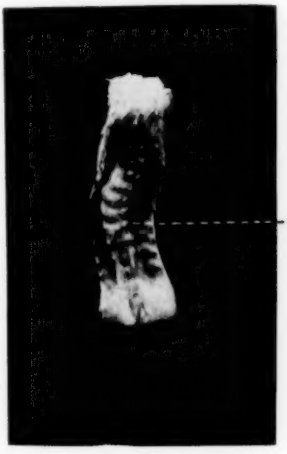

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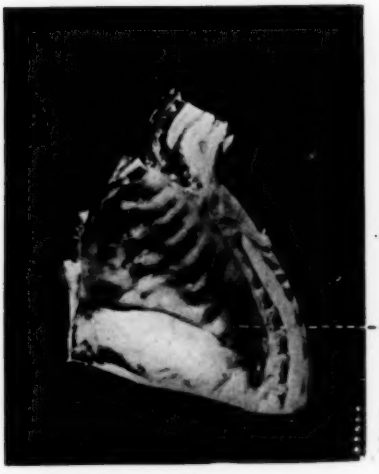

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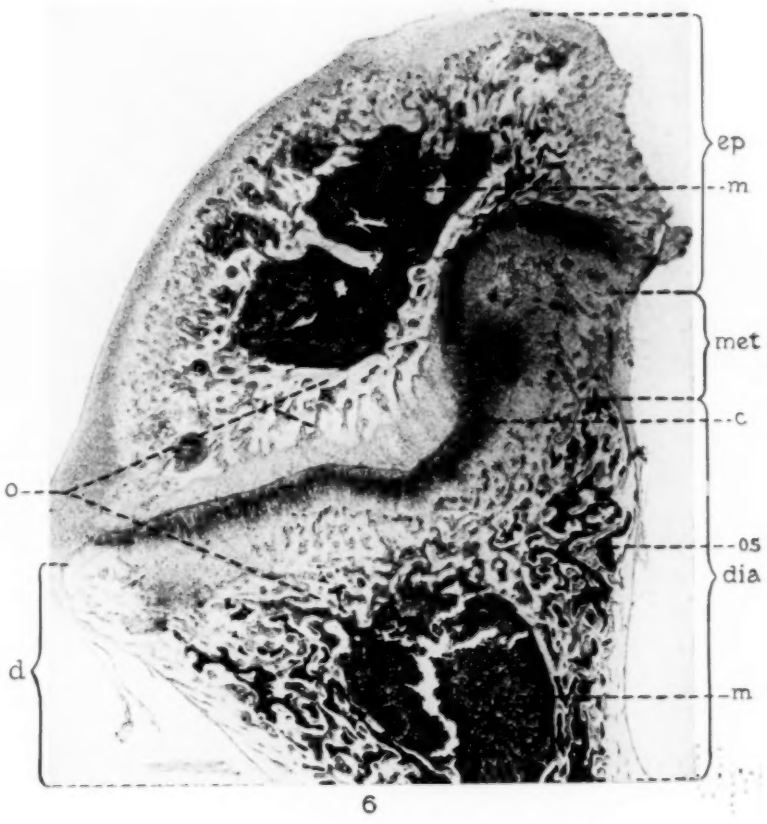
(McCollum, Simmonds, Parsons, Shipley, and Park: Experimental rickets. I.)



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(McCollum, Simmonds, Parsons, Shipley, and Park: Experimental rickets.)

STUDIES ON EXPERIMENTAL RICKETS.

II. THE EFFECT OF COD LIVER OIL ADMINISTERED TO RATS WITH EXPERIMENTAL RICKETS.

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Plates 4 and 5.

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For many years it has been thought as the result of clinical experience that the administration of cod liver oil in rickets exerted a favorable influence on the course of the disease. During the past 15 years studies made on the metabolism of children suffering from rickets has made it reasonably certain that the administration of cod liver oil alters the calcium balance in such a manner that calcium will be retained in the body. The natural inference from both clinical observation and metabolic investigation was that cod liver oil increased the capacity of the skeleton to take up or hold calcium. The object of the experiments reported in this paper was to furnish direct proof that cod liver oil causes calcium salts to be deposited in the bones.

We were already familiar with the observation of Schmorl that, when healing occurs in the rachitic skeleton of the human being, the initial deposition of calcium at the cartilage-shaft junction of the long bones takes place not throughout the rachitic metaphysis or at random in it but on the epiphyseal side of the metaphysis in that zone of the proliferative cartilage in which calcium deposition normally occurs and presumably would have occurred had rickets never been present. Calcium deposits in the cartilage are easy to demonstrate by histological methods and the initial deposit in healing rickets, as seen in the sections taken

longitudinally through the cartilage-shaft junctions, is situated in the lower part of the proliferative zone of cartilage. The lime salts are laid down along a line crossing the bone at right angles to its longitudinal axis and roughly paralleling the upper border of the proliferative zone, forming in the properly stained specimen a picture which cannot well be mistaken. Previous experience with the rat had taught us that by the use of faulty diets, especially certain diets deficient in the so called fat-soluble A or in both that substance and calcium, the cartilage and adjacent portions of the metaphysis of the long bones of the extremities could be rendered entirely free from calcium deposits and a condition identical with the rickets of human beings be obtained. A mode of approach to the problem at issue therefore appeared clear. If cod liver oil possessed calcium-depositing properties there seemed every reason to suppose that when added to the faulty diets of the rats whose epiphyseal cartilages and adjacent portions of the metaphyses had been rendered calcium-free, a lime salt deposit would be produced so characteristic as to have almost specific significance.

With the object of putting cod liver oil to the biological test fifteen young rats were placed on two deficient rations, Nos. 2677 and 2638. Eight animals were fed on Diet 2677 which, as will be seen from the formulas in Table I, is low in the fat-soluble A, and seven were placed on Diet 2638 which is a formula low in Ca, Na, and Cl ions, as well as in fat-soluble A. The administration of these diets it was hoped would render cartilage and the adjacent portion of the metaphysis entirely free from calcium salts.

The young rats listed as having received Diet 2638 may be said to have been on a deficient formula from their birth since the mother rats were maintained on the defective diet throughout the major part of the lactation period. Both lots of rats were maintained for from 50 to 80 days on the faulty rations.

Following the administration of the faulty diets the experimental animal ceased to gain, began to lose weight, and became extremely irritable, reacting to any stimulus with violent activity. About 40 days after the commencement of the administration of the faulty rations it was noted that those animals on Diet 2677 were beginning to develop xerophthalmia, which is characteristic for animals fed on diets deficient in fat-soluble A. When the

condition of the animals had become such as to make it evident that life could be sustained only a few days longer, they were considered ready for the test administration of cod liver oil. To the food of four of the rats on Ration 2677, cod liver oil was added in such proportion that 2 per cent replaced 2 per cent of

TABLE I.

No. and sex of rat.	Diet and principal deficiency.	Age when killed.	Deficient diet administered.	Cod liver oil added to diet.	Weight after death.	Calcium deposit in cartilage
	per cent	*days*	*days*	*days*	*gm.*	
248 ♀	Ration 2638:	75	70	5	49	+
249 ♀	Whole wheat kernel..... 30.0	75	75		66	0
250	" maize " 30.0	64	64			0
251	Polished rice..... 10.0	65	62	3	25	+
252	Rolled oats..... 10.0	67	67		31	0
253	Peas..... 10.0	67	62	5	43	+
254	Navy beans..... 10.0					
	Low in calcium, sodium, and chlorine and also poor in fat-soluble A. When cod liver oil was added to the diet 1 per cent replaced 1 per cent of maize.					
277 ♂	Ration 2677:	100	56		82	0
278 ♀	Rolled oats..... 40.0	110	41	2	64	+
298 ♀	Flaxseed oil meal..... 8.3	125	51	4	77	+
299 ♀	NaCl..... 1.0	110	68		61	0
306 ♂	$CaCO_3$..... 1.5	125	76		80	0
307 ♂	Dextrin..... 49.2	118	68	8	75	+
325 ♂	Low in the fat-soluble A. When	160	84		125	0
326 ♂	cod liver oil was added to the diet 2 per cent replaced 2 per cent of dextrin.	160	77	7	114	0

the dextrin in the ration. This modified formula was fed to the four animals for 2, 4, 7, and 8 days, respectively. To the food of the other four rats no cod liver oil was added and they were preserved as control animals. Of the seven rats on Ration 2638, three were selected as test animals. Cod liver oil was added to

the food of these three so that 1 per cent replaced 1 per cent of maize and the three rats were fed on this formula for 3, 5, and 5 days, respectively. In the case of the four rats remaining which were being fed on Ration 2638 the original diet was continued unchanged. These rats also served as control animals for this diet. As the period of the test administration of cod liver oil came to an end the test animals and their respective controls were killed with chloroform and examined. The bones of test and control animals were compared for gross differences. The ribs, the lower ends of the femora, the upper ends of the tibiæ, the lower ends of the radii and ulnæ, and the upper ends of the humeri were then split longitudinally, placed in 10 per cent formaldehyde, and saved for histological examination. After fixation had taken place the lower end of the femur of each animal was sectioned with a freezing microtome without decalcification, stained with silver nitrate according to the method of von Kossa, and counterstained with hematoxylin and eosin. Blocks of tissue from the ends of all the bones mentioned were embedded in parlodion in some instances without decalcification, in others after short decalcification in Muller's fluid, and stained according to the silver nitrate, hematoxylin, and eosin methods just mentioned.

Microscopic study of the preparations so made permits the following generalizations: When the cartilage has become free from calcium as the result of the deficient diets, the addition of cod liver oil to the food for a period of from 2 to 7 days is followed by deposition of lime salts between the cells of the proliferative zone of cartilage. The deposit of calcium salts is linear, the width of the line apparently depending on the length of time during which the animal has been fed cod liver oil. The line of deposition is at right angles to the long axis of the shaft of the bone. The granular deposit seen in sections stained with hematoxylin does not invade the protoplasm of the cell body. Stained with silver nitrate the granules are colored dark brown or black. It is difficult to say whether or not there is a coincident deposition of calcium in the osteoid tissue of the shaft but from the appearance of the section it would seem that very little lime salt is laid down in the osteoid tissue until calcification of the proliferative cartilage is complete. No gross differences between the bones of the test and control

animals were ever detected, and it was only upon microscopic examination that it was possible to note that in animals which had received no cod liver oil the epiphyseal cartilages were calcium-free. Those to whose diet the oil had been added showed a line of freshly laid down lime salt in the matrix of the proliferative zones. It will be seen from Table I that the cartilages and metaphyses of the bones of all the control animals on both diets used were successfully freed from lime salt deposits and that the administration of cod liver oil caused calcification of the matrix of the proliferative zones of cartilages of all rats fed on Diet 2638. Of the four rats which received cod liver oil after maintenance on Diet 2677 three showed calcification of the proliferative zone and one (No. 326) failed to react thus to the stimulus of the oil administration. It is difficult to assign a reason for the failure of this animal to react to the therapeutic agent but we are quite certain that the cause is to be sought with the animal rather than with the reagent itself. In a series of experiments such as those reported here there are always certain variable conditions encountered which are difficult of control. From among these might be cited variability in appetite or the presence of an absolute anorexia which is always a possibility to be reckoned when dealing with animals in poor physical condition. Again it is possible to bring an animal so near to death by prolonged feeding on a deficient diet that the organism may be without power to respond to remedial substances when they are finally received. These and other factors must be borne in mind in weighing the results of feeding experiments of this type, since the animals used must of necessity be in very poor general health before the restorative treatment is commenced.

The results of this series of preliminary experiments are twofold. They afford ocular and conclusive evidence of the specific beneficial effect of cod liver oil on rats suffering with experimental rachitis, in that some substance or substances in the oil cause calcium to be deposited in the same fashion in which deposition occurs in spontaneous healing of rachitis in man. Moreover, they prepare the way for the elaboration of a new test, which it is to be hoped may eventually prove even to be roughly quantitative, for the determination of the calcium-depositing potentiality of any substance in terms of cod liver oil units. It is

especially interesting to note in the present experiments that calcium was deposited in the cartilages following the initiation of the cod liver oil treatment in spite of the fact that in some cases the calcium intake was far below normal. In view of the fact that in human cases of rickets the blood calcium is maintained throughout the disease at approximately the normal level it is interesting to speculate concerning the source from which the calcium deposited in the proliferative cartilage is derived.

This paper is in all senses a preliminary one and experiments are now under way with a view to perfecting the biological test here reported and to determining the calcium-depositing power of certain other possible therapeutic agents.

EXPLANATION OF PLATES.

ep, epiphysis; *d*, shaft; *met*, metaphysis; *dia*, diaphysis; *m*, bone marrow; *c*, epiphyseal cartilage; *ca*, calcified intracellular substance; *o*, osteoid tissue; *os*, calcified bone.

These photomicrographs were taken of sections cut from frozen tissue previously fixed in 10 per cent formaldehyde. The bone was not decalcified and the sections were stained by von Kossa's method and counterstained with hematoxylin and eosin. Calcified tissue is seen in the photographs as in the section colored black. × 250.

PLATE 4.

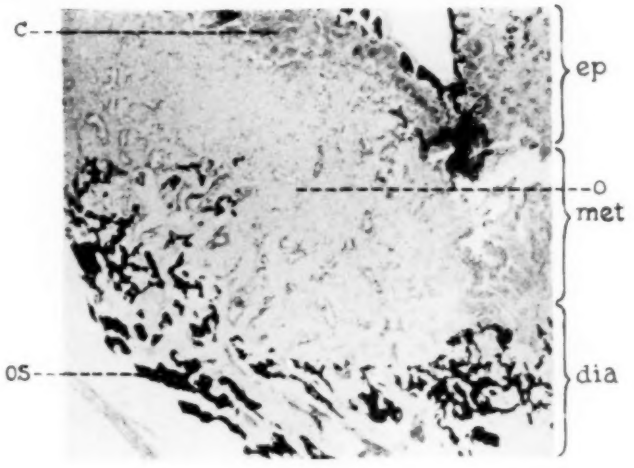
FIG. 1. Sagittal section through the lower ends of the femur of a rat fed for 84 days on Diet 2677. This picture shows besides complete absence of lime salts in the proliferative cartilage, the other cardinal microscopic signs of rickets; *viz.*, invasion of the cartilage and overproduction of osteoid tissue.

FIG. 2. Sagittal section from the distal end of the femur of a rat which was given cod liver oil after being maintained on Diet 2677. Beginning healing of experimental rickets is evidenced by the deposit of calcium (*ca*) in the proliferative zone of the epiphyseal cartilage, otherwise the bone is in the same condition as that in Fig. 1.

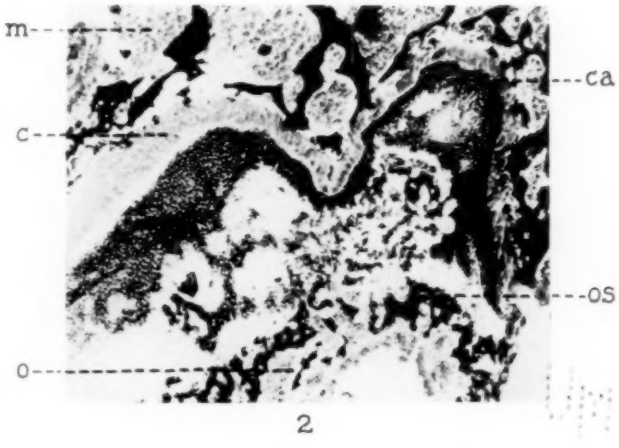
PLATE 5.

FIG. 3. The animal from which the section shown here was made had lived on Diet 2638. The section shows marked evidences of rickets. The cartilage of the epiphysis is absolutely free from calcium salts.

FIG. 4. The rat from which this bone was taken had been given Diet 2638. *Five* days before death 1 per cent of cod liver oil was substituted for 1 per cent of maize in the ration. Note the heavy deposit of calcium (*ca*) in the epiphyseal cartilage resulting from cod liver oil administration.

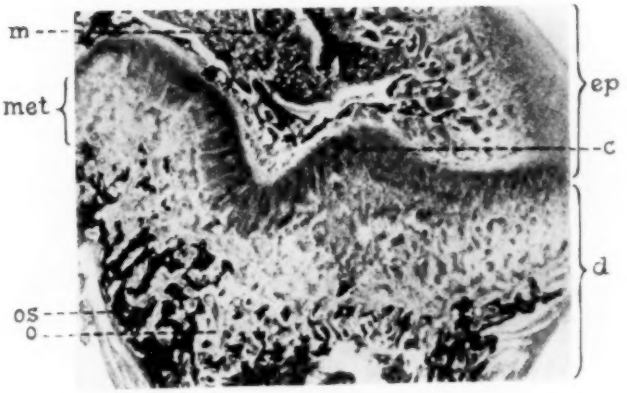


1



2

(Shipley, Park, McCollum, Simmonds, and Parsons: Experimental rickets. II.)



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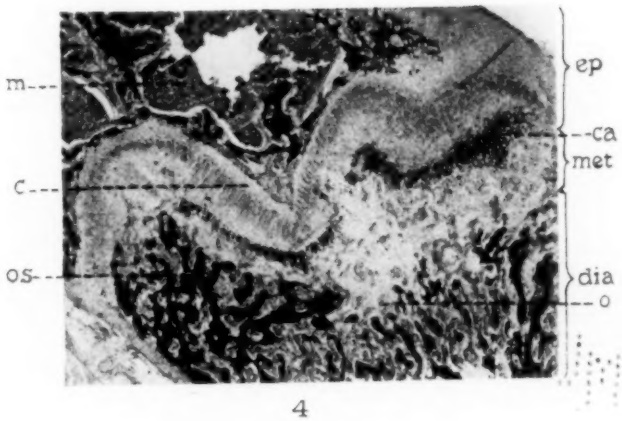

4

THE IODOMETRIC DETERMINATION OF COPPER AND ITS USE IN SUGAR ANALYSIS.

I. EQUILIBRIA IN THE REACTION BETWEEN COPPER SULFATE AND POTASSIUM IODIDE.

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In the course of a comparative study of several methods for the determination of sugar in blood, the results of which will be recorded elsewhere, it became desirable to investigate the reaction between copper sulfate and potassium iodide which is concerned in a number of methods for sugar determination (Bang (1), Maclean (2), Scales (3), Lehmann (4), Riegler (4), Peters (4), Kendall (5)).

In certain of these methods (Bang, Maclean, Scales) the cuprous oxide formed by the oxidation of the sugar is dissolved in acid[1] and reoxidized to cupric salt by a known amount of standard iodine—potassium iodide, the excess of iodine being then titrated with thiosulfate. The oxidation takes place in the presence of considerable excess of cupric salts which under the conditions does not appear to inhibit the oxidation, the latter going apparently to completion.

Essentially the same reaction between cupric salts and potassium iodide is the basis of the well known method for the iodometric determination of copper introduced by De Haen in 1854, the details of which have been studied especially by Gooch and Heath (6) who give the earlier literature. But in this case cupric salt is reduced to cuprous iodide in the presence of an excess of potassium iodide, with the liberation of free iodine

[1] In Bang's micro method the cuprous salt is held in solution by the presence of a large amount of potassium chloride.

equivalent to the amount of copper reduced. Under the conditions laid down by Gooch and Heath the reduction is complete, and correct results are obtained.

This latter reaction, the reduction of cupric to cuprous salt, also has been utilized in the determination of sugar, the excess of cupric copper, after filtering off the cuprous oxide, being determined in this way in methods described by Lehmann, Riegler, Peters, and others (4).

Both procedures outlined above are based upon the reversible reaction

$$Cu^{++} + 2I^- \longleftrightarrow Cu^+ + I^- + \tfrac{1}{2}I_2$$

which under slightly different conditions appears to run, for practical purposes, to completion in either direction. It has long been known that this reaction is reversible and it has been shown by Bray and MacKay (7) to obey the mass law within certain limits in dilute solutions. It therefore seemed rather remarkable that conditions should exist which permit the complete removal of free iodine, one of the active factors in the reaction without perceptibly disturbing the equilibrium at *both* extremes.

In order to permit an intelligent use of the reaction in both directions we have undertaken the determination of the points of equilibria of the reaction

$$CuSO_4 + KI \longleftrightarrow CuI + I_2 + K_2SO_4$$

at various initial concentrations of $CuSO_4$ and KI and at various ratios of $CuSO_4$: KI. Our results indicate the conditions under which the reaction may be utilized for the determination of either cupric or cuprous salts with ease and accuracy.

The experimental procedure was to mix the solutions of copper sulfate and potassium iodide in definite molecular ratios at widely varying concentrations. After standing at room temperature samples of the supernatant liquid (which contained the liberated iodine, unreduced cupric copper, excess potassium iodide, and a minute quantity of cuprous iodide in solution) were carefully pipetted out, and the extent of the reduction, and thus the equilibrium attained, was determined by titrating the amount of free iodine by standard thiosulfate solutions. Since according to the reaction

$$Cu^{++} + 2I^- \longleftrightarrow (CuI) + \tfrac{1}{2}I_2$$

1 atom of iodine is liberated for each atom of copper which is reduced, one may readily calculate the amount of cuprous salt formed and this subtracted from the total copper gives the amount of cupric salt unreduced. It was clear, however, that this method of determining the equilibrium point would not work unless the equilibrium were in some way stabilized; for, on removal of iodine by titration, the equilibrium is rapidly shifted to the right by the now unbalanced cupric and iodide ions; the blue starch-iodide end-point returns very rapidly; and the results indicate much too great reduction. This was avoided simply by measuring the samples for analysis directly into an equal volume of water containing an excess of sodium hydroxide. The cupric copper which remains is precipitated as the hydroxide and removed by filtration while the iodine is converted into hypoiodite and iodide and is recovered on acidifying the filtrate.

With both cupric and cuprous salts[2] removed the reaction cannot take place, and the equilibrium attained is represented by the amount of iodine, which after acidification is titrated with thiosulfate. Under these conditions the end-point is sharp. The results are given in Table I. The details of procedure and the method of calculating the results are given below.

Preparation of $CuSO_4$-KI Mixtures.

An approximate 1.25 M solution was made of Kahlbaum's "Zur Analyse" $CuSO_4.5H_2O$. After filtering from suspended particles this was carefully standardized by titrating 20 cc. portions of a 1:10 dilution with 0.1 N thiosulfate after adding KI under the conditions described by Gooch and Heath. From the original $CuSO_4$ solution, an exactly molecular solution was made by dilution. The concentration of this solution was checked by diluting 50 cc. to 500 cc., when 20 cc. portions titrated with thiosulfate gave 20.05 cc. $\times$ 0.0997 N = 19.99 cc. 0.1 N. Potassium biiodate was used to standardize the thiosulfate. From the M $CuSO_4$, other dilutions were made as needed.

[2] The amount of cuprous iodide in solution is negligible except in the presence of high concentrations of potassium iodide. Bodländer and Storbeck (8) calculate the solubility product of $(Cu^+).(I^-)$ as 5×10^{-12} according to which value the concentration of cuprous salt in dilute solutions of potassium iodide would be less than 2.2×10^{-6} M.

TABLE I.

Equilibria of $CuSO_4 + KI$.

Experiment No.	Initial concentration of $CuSO_4$.	Initial concentration of KI.	Thiosulfate titration of 10 cc. of mixture.			At equilibrium.	
			Found at equilibrium.	Calculated for complete reduction to CuI.	Thiosulfate.	Cuprous salt.	Cupric salt.
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
	millimolar	*millimolar*				*per cent*	*per cent*
			$1CuSO_4$:1KI				
1	2.5	2.5	0.00	25.0	0.001 N	0.00	100.00
2	5.0	5.0	0.00	50.0	0.001 "	0.00	100.00
3	6.0	6.0	0.03	60.0	0.001 "	0.05	99.95
4	7.0	7.0	0.05	70.0	0.001 "	0.07	99.93
5	8.0	8.0	0.15	80.0	0.001 "	0.19	99.81
6	9.0	9.0	0.50	90.0	0.001 "	0.56	99.44
7	10.0	10.0	0.80	100.0	0.001 "	0.80	99.20
8	11.0	11.0	1.20	110.0	0.001 "	1.09	98.91
9	12.0	12.0	1.80	120.0	0.001 "	1.50	98.50
10	20.0	20.0	19.75	200.0	0.001 "	9.88	90.12
11	50.0	50.0	11.74	50.0	0.01 "	23.48	76.52
12	100.0	100.0		100.0	0.01 "	36.50	63.50*
13	200.0	200.0		200.0	0.01 "	40.40	59.60*
			$1CuSO_4$: 2KI				
14	2.5	5.0	0.00	25.0	0.001 N	0.00	100.00
15	3.0	6.0	0.00	30.0	0.001 "	0.00	100.00
16	4.0	8.0	0.20	40.0	0.001 "	0.50	99.50
17	5.0	10.0	0.50	50.0	0.001 "	1.00	99.00
18	6.0	12.0	1.00	60.0	0.001 "	1.67	98.33
19	7.0	14.0	0.25	7.0	0.01 "	3.57	96.43
20	8.0	16.0	0.50	8.0	0.01 "	6.25	93.75
21	9.0	18.0	0.75	9.0	0.01 "	8.33	91.67
22	10.0	20.0	1.10	10.0	0.01 "	11.00	89.00
23	11.0	22.0	1.50	11.0	0.01 "	13.64	86.36
24	12.0	24.0	2.00	12.0	0.01 "	16.67	83.33
25	13.0	26.0	2.40	13.0	0.01 "	18.46	81.54
26	14.0	28.0	2.85	14.0	0.01 "	20.36	79.64
27	15.0	30.0	3.60	15.0	0.01 "	24.00	76.00
28	16.0	32.0	4.18	16.0	0.01 "	26.13	73.87
29	17.0	34.0	4.58	17.0	0.01 "	26.94	73.06
30	18.0	36.0	5.15	18.0	0.01 "	28.61	71.39
31	19.0	38.0	5.80	19.0	0.01 "	30.53	69.47

* Determination of precipitated cupric hydroxide.

TABLE I—*Continued.*

Experiment No.	Initial concentration of $CuSO_4$.	Initial concentration of KI.	Thiosulfate titration of 10 cc. of mixture.			At equilibrium.	
			Found at equilibrium.	Calculated for complete reduction to CuI.	Thiosulfate.	Cuprous salt.	Cupric salt.
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
	millimolar	*millimolar*				*per cent*	*per cent*
			$1CuSO_4$:2KI				
32	20.0	40.0	6.45	20.0	0.01 N	32.25	67.75
33	30.0	60.0	12.35	30.0	0.01 "	41.17	58.83
34	40.0	80.0	19.18	40.0	0.01 "	47.95	52.05
35	50.0	100.0	2.62	5.0	0.10 "	52.40	47.60
36	100.0	200.0		10.0	0.10 "	67.25	32.75*
37	200.0	400.0		20.0	0.10 "	72.50	27.50*
38	300.0	600.0		30.0	0.10 "	75.50	24.50*
			$1CuSO_4$:3KI				
39	2.5	7.0	0.00	25.0	0.001 N	0.00	100.00
40	5.0	15.0	0.35	5.0	0.01 "	7.00	93.00
41	10.0	30.0	3.30	10.0	0.01 "	33.00	67.00
42	20.0	60.0	11.62	20.0	0.01 "	58.10	41.90
43	50.0	150.0	3.87	5.0	0.10 "	77.40	22.60
44	100.0	300.0	9.07	10.0	0.10 "	90.70	9.30
45	200.0	600.0	19.17	20.0	0.10 "	95.85	4.15
46	400.0	1,200.0	38.69	40.0	0.10 "	96.72	3.28
			$1CuSO_4$:4KI				
47	2.5	10.0	0.25	25.0	0.001 N	1.00	99.00
48	5.0	20.0	0.90	5.0	0.01 "	18.00	82.00
49	10.0	40.0	5.47	10.0	0.01 "	54.70	45.30
50	20.0	80.0	14.77	20.0	0.01 "	73.85	26.15
51	50.0	200.0	4.62	5.0	0.10 "	92.40	7.60
52	100.0	400.0	9.77	10.0	0.10 "	97.70	2.30
53	200.0	800.0	19.67	20.0	0.10 "	98.35	1.65
54	400.0	1,600.0	39.57	40.0	0.10 "	98.93	1.07
			$1CuSO_4$: 5KI				
55	2.0	10.0	0.00	20.0	0.001 N	0.00	100.00
56	3.0	15.0	0.85	30.0	0.001 "	2.83	97.17
57	4.0	20.0	3.88	40.0	0.001 "	9.70	90.30
58	5.0	25.0	10.00	50.0	0.001 "	20.00	80.00
59	6.0	30.0	18.25	60.0	0.001 "	30.42	69.58
60	8.0	40.0	33.95	80.0	0.001 "	42.44	57.56
61	10.0	50.0	5.77	10.0	0.01 "	57.70	42.30

TABLE I—*Concluded.*

Experiment No.	Initial concentration of $CuSO_4$.	Initial concentration of KI.	Thiosulfate titration of 10 cc. of mixture.			At equilibrium.	
			Found at equilibrium.	Calculated for complete reduction to CuI.	Thiosulfate.	Cuprous salt.	Cupric salt.
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
	millimolar	*millimolar*				*per cent*	*per cent*
			$1CuSO_4$:5KI				
62	20.0	100.0	17.00	20.0	0.01 N	85.00	15.00
63	30.0	150.0	26.72	30.0	0.01 "	89.06	10.94
64	40.0	200.0	3.70	4.0	0.10 "	92.50	7.50
65	50.0	250.0	4.80	5.0	0.10 "	96.00	4.00
66	60.0	300.0	5.78	6.0	0.10 "	96.33	3.67
67	100.0	500.0	9.77	10.0	0.10 "	97.70	2.30
68	200.0	1,000.0	19.72	20.0	0.10 "	98.60	1.40
69	300.0	1,500.0	29.56	30.0	0.10 "	98.53	1.47
70	400.0	2,000.0	39.34	40.0	0.10 "	98.35	1.65
			$1CuSO_4$:10KI				
71	1.0	10.0	0.00	10.0	0.001 N	0.00	100.00
72	2.5	25.0	6.00	25.0	0.001 "	24.00	76.00
73	3.0	30.0	12.50	30.0	0.001 "	41.67	58.33
74	4.0	40.0	23.90	40.0	0.001 "	59.75	40.25
75	5.0	50.0	3.72	5.0	0.01 "	74.40	25.60
76	8.0	80.0	7.00	8.0	0.01 "	87.50	12.50
77	10.0	100.0	9.57	10.0	0.01 "	95.70	4.30
78	20.0	200.0	19.57	20.0	0.01 "	97.85	2.15
79	50.0	500.0	4.97	5.0	0.10 "	99.40	0.60
80	100.0	1,000.0	9.62	10.0	0.10 "	96.20	3.80
81	200.0	2,000.0	17.62	20.0	0.10 "	88.10	11.90
82	300.0	3,000.0	22.12	30.0	0.10 "	73.73	26.27

A 5 M KI solution was made by dissolving in a volume of 1,000 cc. 830 gm. of a pure preparation, previously powdered and dried at 80°C. for 18 hours. The solution was filtered and from it other dilutions were made. Calibrated pipettes and burettes, the latter of 25 cc. capacity, small bore, and marked in 0.05 cc., were used throughout.

The copper sulfate and potassium iodide solutions were mixed in a uniform way in volumetric flasks. Distilled water was first added to the flasks leaving only slightly more room than needed for the solutions to be added. The $CuSO_4$ was then

added by pipette, the solution mixed, the KI measured in, and finally water added to the mark. After being stoppered and mixed by shaking, the flasks stood in a dark closet at 25–30°C. from 2 to 5 days to insure the attainment of equilibrium. During this time the precipitated CuI settles to the bottom so that it is possible to pipette off samples of the clear supernatant liquid without disturbing the precipitate. 20 cc. samples of the solutions were measured into small flasks containing exactly 20 cc. of N NaOH. After being mixed, the solutions were filtered from the precipitated cupric hydroxide, and 20 cc. of the filtrates, equivalent to 10 cc. of the $CuSO_4$-KI mixtures, were measured into small flasks and acidified with about 5 cc. of 5 N H_2SO_4. The iodine liberated was then titrated with the same thiosulfate (or dilutions made from it) which was used in standardizing the $CuSO_4$ solution (Column 4, Table I).

The use of sodium hydroxide makes necessary the application of a small correction for the amount of iodine which is removed by some impurity in the alkali, the correction being added to the amounts of thiosulfate used. The correction in these experiments amounted to 1.11 cc. of 0.01 N iodine.

At the same time other 10 cc. portions of the supernatant liquids were in most cases titrated directly with thiosulfate after adding an excess of KI. The results so obtained are equivalent to the *total* copper originally in solution and serve as an additional check upon these values. In all but a few cases the results for total copper agreed well with the calculated values.

The following example illustrates the procedure above described.

10 cc. of 1 M $CuSO_4$ were mixed with about 175 cc. of water in a 200 cc. volumetric flask, 10 cc. of 5 M KI added, and the solution was diluted to the mark. The mixture had therefore as the initial concentrations, 50 millimolar Cu and 250 millimolar KI (Experiment 65, ratio $1CuSO_4$:5KI, Table I). After standing 3 days a 20 cc. sample of the supernatant liquid was pipetted out, added to 20 cc. of N NaOH, and filtered.

	0.1 N thiosulfate. cc.
20 cc. filtrate, acidified (= 10 cc. original solution)	4.69
Correction for NaOH	0.11
(Column 4, Table I)	4.80
10 cc. original solution + excess KI = total copper (Column 5)	5.00

Since 1 atom of iodine is liberated from each atom of copper reduced, 10 cc. of 0.05 M $CuSO_4$ when fully reduced by KI should yield 10 cc. of 0.05 N or 5.0 cc. of 0.1 N iodine, which is the amount found by titration. The amount found at equilibrium (4.80 cc.) divided by the amount from total reduction (5.00) gives the proportion reduced (0.96 or 96.0 per cent) (Column 7). The remainder (4 per cent) is the proportion of cupric salt (Column 8).

The procedure of analysis and methods of calculation above described were adhered to throughout, with the following exceptions. In very dilute solutions the iodine liberated is so small in amount that all or most of it was absorbed by the impurity present in the NaOH, the correction for the blank approaching or exceeding the amount of iodine to be determined. In these cases 10 cc. of the supernatant liquid were titrated directly with 0.001 N thiosulfate without previously stabilizing the equilibrium. Fortunately at very low concentrations of copper and iodide the small amounts of iodine present can be titrated away without materially shifting the equilibrium within the brief period of making the titration, and the results of direct titration are moderately reliable as proved by a fair agreement of results by the usual procedure and by direct titrations, at the slightly higher concentrations where such comparison was possible.

Another exception to the procedure described was necessary with the higher concentrations of the series, 1 $CuSO_4$: 1 KI and 1 $CuSO_4$: 2 KI. The results first obtained with the alkaline filtrates from solutions 100 millimolar $CuSO_4$ of series 1:1 and of series 1:2 and from solutions of higher concentrations with these ratios, gave values which on being plotted on curves appeared erroneous. On inspection of the precipitate in the flasks, crystals of iodine were observed to be mixed with the cuprous iodide, which explained the low results obtained. The residual iodide, after the equilibrium was reached, was under these conditions not sufficient to hold in solution all the iodine liberated, and the titration of the iodine in solution therefore did not represent the amount formed. In such cases the points of equilibria were located by determining directly the amount of residual cupric copper. The cupric hydroxide precipitated from the supernatant liquid when the latter is run into NaOH was washed free of iodine by repeated centrifugation, decantation, and filtration,

finally dissolved in H_2SO_4, an excess of KI added, and the solution titrated with thiosulfate under the conditions under which the copper sulfate solutions are standardized. These results proved to be reliable and in agreement with those from the usual procedure in those solutions where such comparison was possible. Data were obtained in this way for the last two mixtures of ratio 1:1 and the last three mixtures of ratio 1:2 as explained in the foot-note to Table I.

DISCUSSION.

From the data given in Table I curves have been drawn which show graphically the position of the equilibria under various conditions. In Charts 1 and 2 the percentage of total copper reduced to cuprous salt (the position of equilibrium) is plotted against initial concentration of total copper for each of the molecular ratios 1 $CuSO_4$:1, :2, :3, :4, :5, and :10 KI. The initial concentration of KI for each curve is readily seen by multiplying the initial copper concentration by the KI ratio of that curve. In Chart 3 the percentage of total copper reduced is plotted against initial concentration of potassium iodide.

Each individual curve of Chart 1 shows the shift of equilibrium with change in volume for a given molecular ratio of the reacting substances. If a mixture of $CuSO_4$ and KI is made of initial concentrations as indicated and the mixture is progressively diluted the percentage distribution of total copper between precipitated cuprous iodide and cupric salt in solution is shown at any given dilution by the corresponding point on the curve for that molecular ratio. For instance, if equal volumes of 0.2 M $CuSO_4$ and 1.0 M KI are mixed, the initial concentrations being 100 millimolar $CuSO_4$ and 500 millimolar KI (1 $CuSO_4$: 5 KI), about 98 per cent of the total copper is present as cuprous iodide. If the mixture is diluted with 9 volumes of water, the equilibrium is now such that only 57 per cent of the copper exists as cuprous iodide, and on further dilution to 50 times the original volume (total copper 2 millimolar, total KI 10 millimolar) practically all the copper exists as cupric salt and only little if any iodine can be detected by starch.

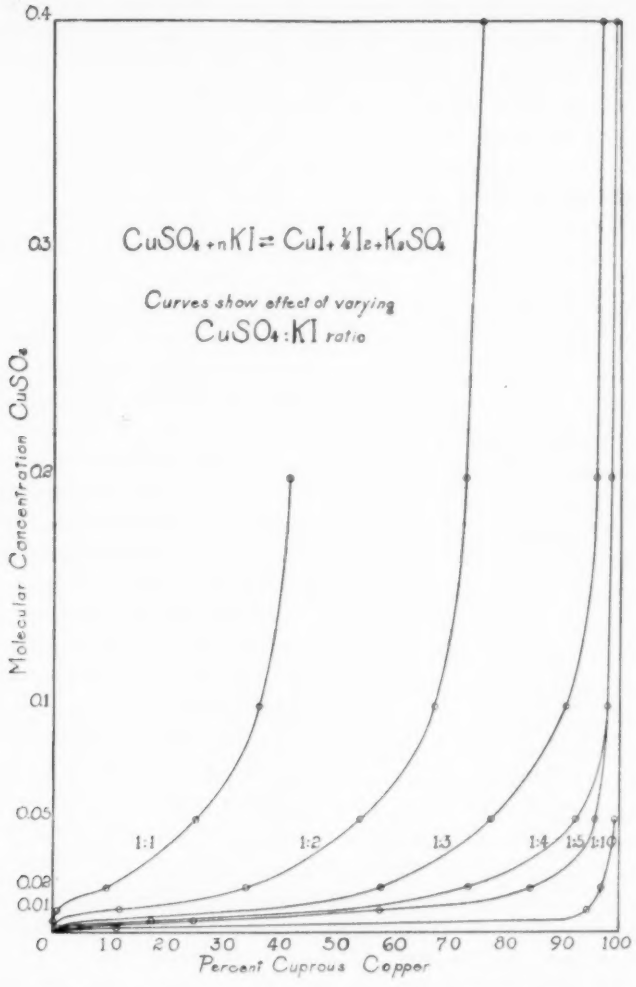

CHART 1. Curves show the *position of equilibrium* of the reaction between $CuSO_4$ and KI, expressed as percentage of total copper in the form of cuprous salt, as affected by varying the ratio of $CuSO_4$:KI, and by concentration (dilution).

The relations are illustrated by the following experiment.

Mix 2.5 cc. of M (25 per cent) $CuSO_4.5H_2O$ and 7.5 cc. of M (16.6 per cent) KI. Cuprous iodide is immediately precipitated and the solution is dark-colored from the formation of free iodine. On analysis 96 per cent of the copper would be found as cuprous iodide. This mixture had initial concentrations of 250 millimolar $CuSO_4$ and 750 millimolar KI or $1CuSO_4$:3KI. Mix the same amounts in a total volume of 1,000 cc. by adding 2.5 cc. of M $CuSO_4$ to 990 cc. of water, and to this dilute solution add 7.5 cc. of M KI. The mixture now remains clear and gives no iodine reaction with starch.[3] The initial concentrations in this case correspond to 2.5 millimolar $CuSO_4$ and 7.5 millimolar KI.[4]

It is evident that the proportion of cupric salt which is reduced to cuprous iodide is determined by the dilution and by the relative excess of iodide in solution. The predominant influence of iodide is indicated by Chart 3. These relations are implied in the mass law equation

$$(Cu^{++}) \cdot (I)^2 = \sqrt{I_2} \cdot K$$

according to which the concentration of cupric ions will vary inversely as the square of the iodide ions and directly as the square root of the free iodine. A discussion of the bearing of our data upon the validity of this equation will be deferred to a later paper, and we shall point out here only their significance for the determination of cupric and cuprous copper, for which purpose the work was undertaken.

[3] Since writing the above the work of Traube (Traube, M., *Ber. chem. Ges.*, 1884, xvii, 1064) has come to our attention. Traube established the existence of cupric iodide in solution and described experiments very similar to those given above showing the effect of dilution upon the formation and precipitation of cuprous iodide from mixtures of copper sulfate and potassium iodide. He pointed out that the mixture of 0.1 per cent KI and 0.075 per cent $CuSO_4.5H_2O$ (equivalent to final concentrations of 3 millimolar KI and 1.5 millimolar $CuSO_4$) or 0.05 per cent KI with 1.0 per cent $CuSO_4.5H_2O$ (equivalent to final concentrations of 1.5 millimolar KI and 20 millimolar $CuSO_4$) remains clear and gives no reaction with starch after 24 hours standing. Our work is thus an elaboration of facts made known 36 years ago.

[4] The same experiment may be more simply performed by diluting the first mixture to a liter, but in this case equilibrium is attained more slowly and the solution contains a small amount of free iodine for some time.

With the ratio of 1 Cu : 1 KI reduction is absent or very slight until the concentration reaches about 5 millimolar (Chart 2). With these relative amounts of cupric salt and potassium iodide and at these concentrations no cuprous salt and no free iodine are formed. Furthermore, if cuprous salt is originally

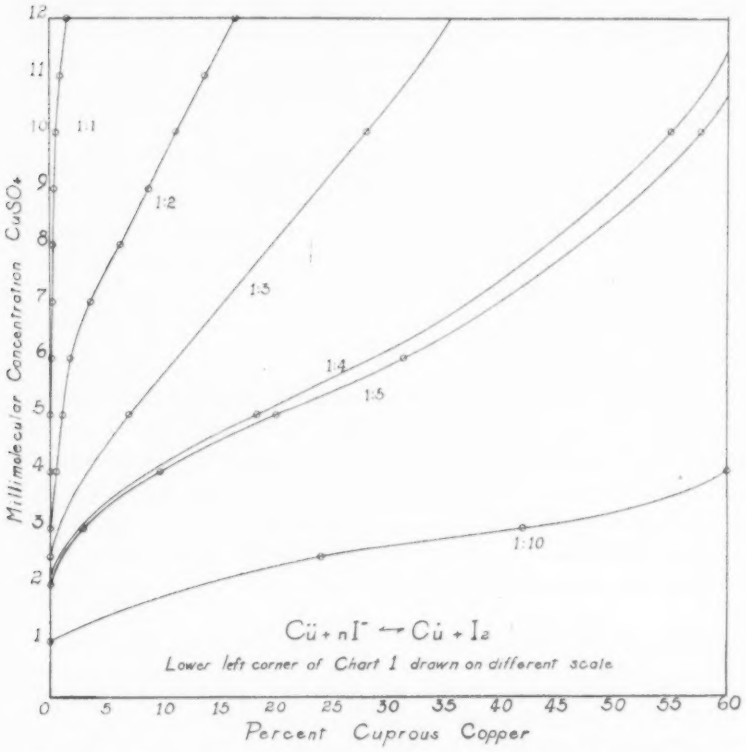

CHART 2. Curves show the same relations as in Chart 1, drawn on a larger scale to indicate the limiting concentrations for the complete oxidation of cuprous to cupric salt.

present it is quickly and completely oxidized to the cupric condition if iodine is added; and since at this concentration there is no tendency for the reaction to run to the right, and thus no cuprous salt and free iodine to form, the excess iodine may be removed by titration with a sharp end-point. The

conditions, therefore, which must be chosen for the determination of cuprous copper by this reaction are a minimum excess of KI and a final (total) concentration of less than about 3 to 5 millimolar copper. (In the following paper means are described by which this limitation is removed.) Cuprous copper will be completely oxidized to cupric salt by an excess of iodine provided the final concentration of copper and iodide do not exceed 5 millimolar each.[5]

These are the conditions which Maclean and Scales found empirically to be necessary for the reoxidation by iodine of cuprous copper formed in the oxidation of sugar. The final concentration of total copper in Maclean's titration is 2.5 millimolar and of iodide 2.9 millimolar (after the amount of iodide necessary for the reaction with the iodate has been subtracted from the original amount present) or 1:1 ratio. In the technique described by Scales the values prove to be 4.5 millimolar iodide and 2.3 millimolar total copper or 1:2 ratio. An examination of Chart 2 shows that both these conditions lie on the ordinate below the intersection of Curves 1:1 and 1:2, where no cuprous salt is present at equilibrium. At concentrations represented by points above the intersection of the respective curves, the end-points would not be sharp and the results would be incorrect. At such higher concentrations a large excess of iodine will, to be sure, oxidize more or less cuprous salt but on removal of the excess of iodine during titration with thiosulfate the equilibrium will shift to the right with the reformation of cuprous iodide and iodine and a consequent running (returning) end-point.

[5] The actual limitation is doubtless a certain value of the product $(Cu^{++}) \times (I^-)^2$. What this value is we have not determined exactly though our results indicate it to be roughly about 135, the concentrations being expressed in terms of millimolar solutions.

Ratio 10:1,	1 Cu ×	(10 I)²	= 100
" 5:1,	2 Cu ×	(10 I)²	= 200
" 4:1,	2.1Cu ×	(8.2I)²	= 141
" 3:1,	2.5Cu ×	(7.5I)²	= 140
" 2:1,	3.0Cu ×	(6 I)²	= 101
" 1:1,	5 Cu ×	(5 I)²	= 125
Average			135

For the determination of cupric copper, on the other hand, it is necessary that the cupric salt be completely reduced with the liberation of an equivalent amount of iodine, and, as shown by Chart 1, this condition depends upon the presence of a considerable excess of iodide in solution. The optimum concentration of iodide is about 0.25 to 0.5 M, in which solutions, if the ratio of

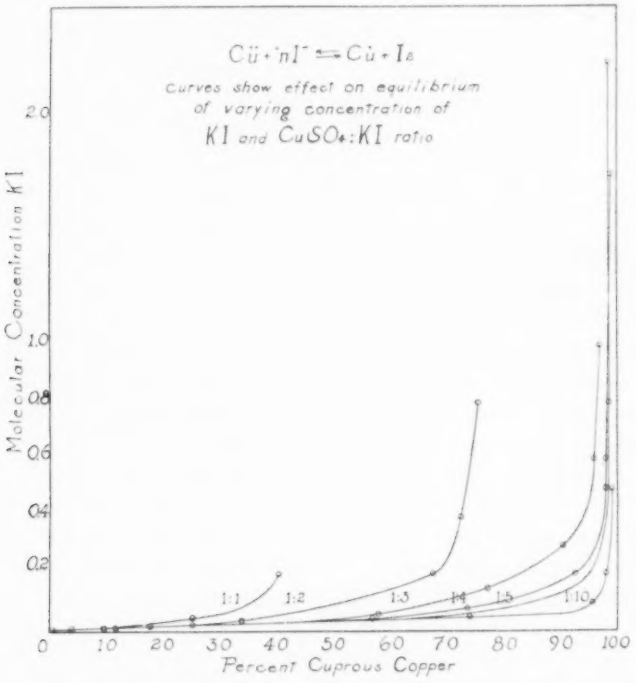

CHART 3. The curves show the predominating influence of KI in determining the equilibrium.

copper to iodide is not greater than 1:5, the copper will be almost completely converted into cuprous iodide at equilibrium, and on removal of the iodine by titration the small remainder of cupric salt is quickly reduced. Equilibrium being close to the right side of the reaction, the titration end-point is sharp. In a volume of 100 cc., 0.25 M potassium iodide (4.2 gm.) allows the correct determination of amounts of copper up to the equivalent of

1.25 gm. of $CuSO_4 . 5 H_2O$, or double this amount with 0.5 M KI. With smaller concentrations of iodide, as is apparent from Chart 1, the reduction is less complete at equilibrium, and the titration end-points are in consequence less sharp, the blue color returning as long as cupric salts are present.

It is doubtless due to the lack of such information as may be derived from Chart 1 that varying opinions have been expressed as to the value of this method for the determination of copper (cited by Gooch and Heath (6)). Those who have favored the method adopted conditions the correctness of which are confirmed by our curves. Moser (9) recommends the addition of 2 gm. of potassium iodide to 50 cc. of a solution containing about 0.6 gm. of cupric sulfate; this is equivalent to about 0.24 M iodide and at least 5 KI:1 Cu. Gooch and Heath recommend the use of 5 gm. of potassium iodide in 100 cc. of solution containing not more than 0.3 gm. of copper. These amounts are equivalent to 0.3 M iodide and at least 6 KI:1 Cu.

SUMMARY.

Both cupric and cuprous salts may be determined iodometrically by means of the reversible reaction

$$Cu^{++} + I^- \longleftrightarrow Cu^+ + I_2$$

The position of equilibrium has been determined for the reaction

$$CuSO_4 + n\,KI = CuI + I_2 + K_2SO_4$$

at ratios of from 1 to 10 KI : $1CuSO_4$ at various dilutions.

For the determination of cupric salts potassium iodide must be added to give a *final* concentration of about 0.25 M (4 to 5 gm. per 100 cc. of solution).

For the determination of cuprous salts the solution must be so diluted that the *final* concentration of copper and of iodide does not exceed about 5 millimolar each.

The conditions found empirically by Gooch and Heath (6) for cupric salts and by Maclean (2) and Scales (3) for cuprous salts comply with these requirements.

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THE IODOMETRIC DETERMINATION OF COPPER AND ITS USE IN SUGAR ANALYSIS.

II. METHODS FOR THE DETERMINATION OF REDUCING SUGARS IN BLOOD, URINE, MILK, AND OTHER SOLUTIONS.

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After heating Fehling's solution or similar alkaline copper solution with a reducing sugar, the solution contains the oxidation products of the sugar, an excess of cupric salt, and suspended cuprous oxide. One may learn the amount of sugar oxidized by determining either the cuprous oxide, or, knowing the total copper, the residual cupric salt. Both forms of copper have been determined by means of iodometric titrations, as illustrated by the procedures of Lehmann (1), Riegler (2), Maquenne (3), Citron (4), and Peters (5) for residual cupric salts and of Bang (6), Scales (7), Maclean (8), and Clark (9) for cuprous salts. The principle underlying these methods and the conditions necessary in the determination of both forms of copper are more exactly defined in the preceding paper (10).

In one case the residual cupric salt is completely converted into cuprous iodide, with the liberation of an equivalent amount of iodine

$$2\,Cu^{++} + 4\,I^- \rightarrow 2\,CuI + I_2$$

in the other case cuprous salt is completely oxidized to cupric in the presence of a known excess of iodine, with the conversion of the corresponding amount of iodine into iodide

$$2\,Cu^{++} + 2\,I^- \leftarrow 2\,Cu^{+} + I_2$$

The iodine formed from iodide in the first case, and the excess iodine left in the second case, are determined by titration with standard thiosulfate, starch being used as indicator.

The direction in which this reversible reaction will proceed depends upon the concentration of the active substances (or ions). For the complete conversion of cupric to cuprous salt the concentration of iodide, which has much the strongest influence, must be high, the optimum being about 0.25 M.

For the complete oxidation of cuprous salts by iodine on the other hand means must be found for maintaining the concentrations of cupric and iodide ions at very low values. As shown in the preceding paper this is accomplished when the solution is diluted so that the total copper and final iodide concentrations do not exceed about 0.005 M each, the equilibrium then being for practical purposes on the cupric side of the reaction. In this way Bang, Maclean, Scales, and Clark were able to determine very small amounts of cuprous oxide with considerable accuracy, even in the presence of cupric salts and iodide; though none of these workers appears to have been aware of the limiting effect of iodide and copper concentrations.

Great dilution, however, limits the titration of cuprous copper to small amounts and curtails its general usefulness. Fortunately, other means are available for reducing the concentration of cupric ions to such a small value that even at moderate dilution and in the presence of much soluble iodide the reaction does not proceed to the right and equilibrium is maintained with the copper wholly in the cupric form. It was pointed out by Elbs (11) in 1917 that the presence of alkali oxalates more or less completely inhibits the reaction of cupric salts with soluble iodides, as well as other reactions of cupric ions. Elbs interpreted this behavior by the assumption of the formation of alkali cupric oxalate which dissociates with the formation of anions containing oxalate and copper, the cations being those of the alkali. This observation, which is easily verified, permits one to direct at will the above reversible reaction *to completion in either direction*, and at practically any concentration of copper salt. The following experiment illustrates the remarkable effect of oxalate.

To 50 cc. of approximately 0.1 M $CuSO_4$ were added 5 gm. of potassium iodide; the liberated iodine required 47.50 cc. of 0.1058 N thiosulfate or 50.23 cc. of 0.1 N. To the same solution, after titration with thiosulfate, were then added 100 cc. of 0.0969 N iodine (= 96.90 cc. of 0.1 N) and 25 cc. of

molar potassium oxalate. After a moment of shaking the cuprous iodide completely dissolved, and on again titrating with thiosulfate 44.10 cc. = 46.65 cc. of 0.1 N were required, showing that 50.25 cc. (96.90 − 46.65 cc.) of 0.1 N iodine had been used for the oxidation. The two determinations on the same sample are in full agreement, the same amount of iodine being used for oxidation as was liberated in reduction, and thus proving the reoxidation to be substantially complete.

The cupric salt is first completely reduced and precipitated as cuprous iodide in the presence of a large excess of soluble iodide, the liberated iodine being reduced to iodide on titration with thiosulfate. The subsequent addition of more free iodine shifts the equilibrium slightly toward the cupric side, but the concentration of iodide (5 gm. of KI in 200 cc. or about 0.15 M) is such that only about 15 per cent of the total copper can be in the cupric form (see Chart 3 of preceding paper). The addition of the oxalate, however, shifts the equilibrium wholly to the cupric side of the reaction and in spite of the excess of potassium iodide present the cuprous iodide is at once dissolved and oxidized by the free iodine. Equilibrium is attained almost immediately and is not affected by the removal of the excess of free iodine in the second titration with thiosulfate. As a rule the starch blue color does not reappear even on standing several days. The solution is colored blue from the complex copper salt, but the starch blue end-point is remarkably sharp and distinct, and after a single trial is never confused with the color due to the copper salt. After a short time, often before the end of the titration if the solution is cold, a precipitate of cupric oxalate or cupric potassium oxalate may form, but this in no way interferes with the titration.

The same phenomenon may be shown qualitatively as follows.

Add to a solution of copper sulfate (25 cc. of Fehling's copper solution) a few grams of potassium iodide; insoluble cuprous iodide is precipitated and the solution contains much free iodine. Add at least four molecular equivalents (5 gm.) of potassium oxalate, and shake; the cuprous iodide dissolves, the iodine disappears, and the solution assumes the blue color of cupric salts. The addition of starch indicates the presence of little or no free iodine. Or to the copper solution add first the oxalate; the addition of KI now fails to form iodine and cuprous iodide.

The only interpretation of the phenomenon would appear to be that by the formation of the complex copper oxalate the num-

ber of cupric ions is so far reduced that the product of their concentration by the square of the iodide concentration

$$(Cu^{++}) \times (I^-)^2$$

is below the threshold value required to cause the reaction to take place to a degree sufficient for its detection by the starch test for iodine. This threshold value was roughly calculated at about 135[1] according to which the concentration of Cu^{++} ions in the above experiments (approximately 0.06 M copper solution) would be less, *probably very much less*, than 0.000006 M.[2]

Taking advantage of this effect of oxalate one has the choice of two almost equally satisfactory, convenient, and accurate iodometric methods for the determination of the amount of copper reduced by sugar. After acidifying the alkaline copper solution, either the residual cupric salt or the cuprous salt, following their reaction with iodide or iodine respectively may be titrated with standard thiosulfate. The oxidation products of the sugar do not react with iodine *in acid solution* and cause no interference in the reactions. It is unnecessary to remove the cuprous oxide in the determination of cupric salt (Maquenne (3)) as prescribed by Peters and others; nor is it necessary to isolate the cuprous oxide. Either may be accurately determined in the presence of the other. Both methods yield the same results within the error of manipulation and titration. These facts permit the application of the very delicate and accurate methods of iodometry to the determination of reducing sugar under a variety of circumstances. We have used the methods in various forms with sugar solutions, in food analysis, with urine, blood, and milk, and shall describe the procedures which we find most convenient and satisfactory. On a number of points there is room for individual choice and there are doubtless other modifications which would be equally satisfactory.

We shall describe procedures for both the "cupric titration" as we may designate the method based upon the determination of the residual cupric salt by its reaction with an excess of potas-

[1] Shaffer, P. A., and Hartmann, A. F., *J. Biol. Chem.*, 1920–21, xlv, 361, foot-note 5.

[2] The phenomenon is a simple and striking illustration of reversible reactions suitable for class experiments.

sium iodide, and the "cuprous titration" or the determination based upon the reoxidation of cuprous salt by iodine made possible by the effect of oxalate. Both methods have been applied for the determination of either large or small amounts of reducing sugar, different solutions being used for the micro titrations. The cupric titration is essentially that introduced by Lehmann and later modified by Maquenne, Riegler, Peters, and others, while the cuprous titration with the aid of oxalate has not previously been used. Because of the greater cost of the large amounts of potassium iodide required for the cupric titration, and especially the fact that the starch end-point is less easily seen in the presence of the precipitated cuprous iodide and is less permanent, we distinctly prefer the cuprous titration, but both methods are in our experience preferable on the grounds of convenience, speed, and reliability to any other volumetric or colorimetric procedures for the determination of reducing sugar with which we are familiar. The degree of accuracy depends only upon the care in manipulation, especially as to the conditions during the oxidation of sugar.

THE DETERMINATION OF LARGE AMOUNTS OF SUGAR.

With either of the reactions above mentioned, the accurate determination of copper is easily accomplished. But the determination of sugar involves also the amount of copper reduced by a given amount of sugar, and this ratio is known to be affected by the concentration of copper, the alkalinity, the duration of heating, and the extent of oxidation of sugar or reoxidation of cuprous oxide by oxygen dissolved in the solution, all of which factors must be controlled or standardized. Although we have formulated new solutions and special conditions for certain purposes it seemed desirable also to adapt the two iodometric titrations to solutions and conditions of heating now in general use. We have therefore adopted the use of Fehling's Soxhlet solutions, and the amounts and conditions of heating prescribed by Munson and Walker (12), and are thus enabled to use their tables for the conversion of copper into terms of sugar. As shown by the results given in Table I, substantially correct values are obtained by using Munson and Walker's figures. In addition we are using

a carbonate-citrate solution, containing in a single solution the iodine and oxalate required for the cuprous titration, which possesses certain advantages and which also will be described.

TABLE I.

Results of Determination of Pure Glucose Solutions. Fehling's Solutions. Cupric and Cuprous Titrations. Kahlbaum's "K" Glucose and United States Bureau of Standards Glucose, Dried at 85°C. Solutions Made by Weight.

Glucose taken.	Cuprous titration.		Cupric titration.	
	Cu reduced.	Glucose found (Munson-Walker).	Cu reduced.	Glucose found (Munson-Walker).
mg.	*mg.*	*mg.*	*mg.*	*mg.*
50	101.5	49.7		
	100.9	49.4		
100	199.0	100.3		
	199.5	100.5		
	198.4	100.0		
	198.0	99.8		
200	375.0	200.2		
	373.8	199.4		
	374.2	199.6	374.0	199.5
	373.3	199.2	373.6	199.3
	373.4	199.2	374.3	199.6
	376.5	201.0	375.5	200.4

Fehling's Solution.

Method.—Measure 25 cc. (pipette) of each of the two Fehling's Soxhlet solutions[3] into a 300 or 400 cc. flask. Add 50 cc. or less of the (approximately neutral) sugar solution containing from 20 to 200 mg. of sugar, and water (if necessary) to make a total volume of 100 cc. Cover with a small inverted beaker and heat on an asbestos mat over a flame so adjusted as to bring the solution to boiling in 4 minutes. Boil 2 minutes. Stand the flask in the sink under running water till cool (3 to 4 minutes). The subsequent treatment depends upon whether the cuprous or cupric titration is used; we prefer the former.

[3] Fehling's Soxhlet Solution 1: 34.64 gm. $CuSO_4.5\,H_2O$ in 500 cc. Solution 2: 173 gm. Rochelle salt and 50 gm. NaOH in 500 cc.

Cuprous Titration.—Add 50 cc. (accurate pipette) or 25 cc. if only little cuprous oxide is present, of the iodate-iodide solution (see below), followed by 15 to 17 cc. of 5 N H_2SO_4. The acid should be added from a cylinder or fast flowing pipette in order that the acidification of the whole solution be accomplished promptly; hypoiodite in *alkaline* solution oxidizes the organic acids present and some iodine may be thus used if acid is added very slowly. There is no danger of this error if the acid is added quickly. Shake the solution gently for a few moments until the cuprous oxide has dissolved. The solution should become clear, though often some cuprous iodide will separate. Add 20 cc. of saturated potassium oxalate and rotate the flask until the cuprous iodide is *completely* dissolved.

If the alkaline solution is taken from the tap while it is still slightly warm (about 40°) and the iodate, acid, and oxalate are added, the cuprous oxide dissolves almost immediately and the solution remains clear; when cold, cuprous iodide may first separate, and when this is dissolved after the addition of oxalate, acid potassium tartrate may crystallize, but neither affects the result. Titrate the solution with standard thiosulfate (0.1 N), adding a few cc. of starch solution toward the end, before the disappearance of the green color.

It is necessary to know accurately the amount of iodine added in the iodate solution, and the blank reduction of the Fehling's solution. Boil the Fehling's solution with 50 cc. of water instead of sugar, cool, and treat as above described. The value of the blank remains practically unchanged for long periods if the Fehling's solutions are measured separately and thus mixed only as used, and its determination need be made only occasionally.

Calculation.—From the blank titration subtract the titration of the sugar determination, the remainder representing I_2 required for the oxidation of cuprous salt. Multiply by the copper factor of the thiosulfate (1 cc. 0.1 N = 6.36 mg. Cu), and find the amount of sugar equivalent to the copper by reference to Munson-Walker tables (12)[4] or divide the amount of copper reduced by the corresponding ratio obtained from the glucose curve in Chart 1.

[4] See also Mathews, A. P., Physiological chemistry, New York, 1915, and *U. S. Dept. Agric., Bureau of Chemistry, Bull. 107*, 1912.

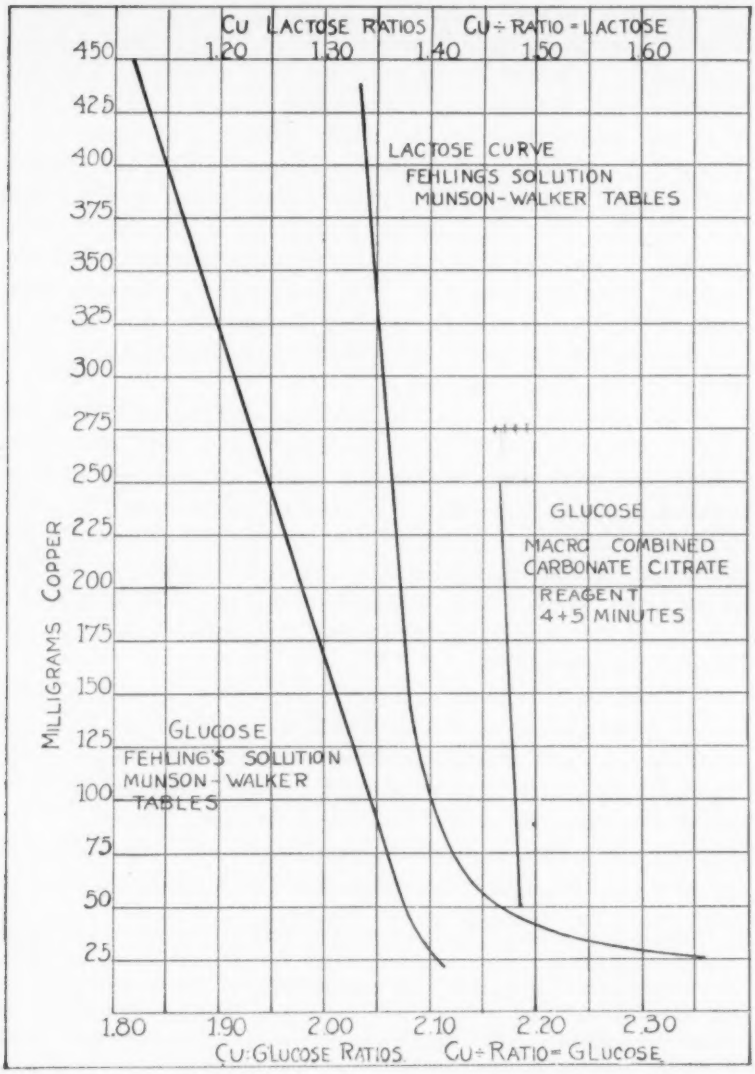

CHART 1. Copper: glucose ratios for Fehling's solution and for the macro combined reagent, and copper:lactose ratios for Fehling's solution. Divide the amount of reduced copper by its corresponding ratio as shown by the respective curves.

Composition of Iodate-Iodide Solution.—5.4 gm. of KIO_3 and 60 gm. of KI are dissolved in water to which is added a small amount of alkali (to prevent the formation of hydriodic acid and its oxidation by air), and diluted to a liter.

Cupric Titration.—To the cooled alkaline copper solution add 6 gm. of KI and 25 cc. of 5 N H_2SO_4. Titrate with standard thiosulfate, adding starch solution toward the end. The titration is subtracted from a similar blank determination on the Fehling's solution, the difference representing copper reduced by the sugar. For the cupric titration the copper solution must be measured accurately. Consult Munson-Walker tables or Chart 1 for the conversion of copper into terms of sugar.

The amount of acid added in both titrations is important. In the cuprous titration an excess sufficient to dissolve the cuprous oxide and to liberate the iodine from the iodate-iodide mixture is essential, but a *very* great excess causes the decomposition of the complex oxalate and is therefore to be avoided. An *excess* of 15 to 40 cc. of N acid over the amount required to neutralize the alkali of the Fehling's solution is satisfactory; this is equivalent to a total of 16 to 20 cc. of 5 N acid. A larger excess of acid is necessary in the cupric titration for the same reason for which it is to be avoided in the cuprous titration. The tartrate and perhaps the organic products of the sugar oxidation tend to form complex salts with cupric copper similar to the oxalate and thus to prevent its complete reduction to cuprous iodide, and these complex salts are unstable in the presence of high acidity. The cupric titration blank is somewhat lower when the solution is only slightly acidified than the value obtained on adding a large excess of acid. Sulfuric acid and not hydrochloric must be used for the neutralization.

Copper-Carbonate Solutions.

The reagent devised by Benedict (13) and widely used for the qualitative detection of sugar in urine has the advantage that, due in part to the lower alkalinity of alkali carbonate used as a substitute for the hydroxide, the reagent does not yield *precipitates* on boiling with dilute solutions of creatinine, uric acid, chloroform, and perhaps other substances which do reduce the

more alkaline Fehling's solution. A further advantage is that the reagent does not deteriorate with age. With these points in mind we have used Benedict's qualitative reagent and a number of similar copper-carbonate solutions for the iodometric determination[5] of glucose in urine and other solutions in the expectation that the normal constituents of urine would not reduce copper at the lower alkalinity. As explained later, however, this expectation is only in part realized.

By the addition to Benedict's or other similar solution of suitable amounts of potassium iodide one may prepare a single reagent of good keeping quality and in some respects satisfactory for sugar determination by "cupric titration" of the residual unreduced copper. Similarly, by adding iodide, iodate, and oxalate to such solutions one may prepare a single permanent reagent for the "cuprous titration;" and we have developed and recommend such a solution, the preparation and use of which are described below. This reagent appears to be permanent in that its blank titration does not change, and its use is rapid, convenient, and accurate as far as concerns the determination of copper, the sharpness of end-point, and agreement of duplicates. It is applicable to the determination of reducing sugar in moderately large amounts (up to 150 mg. of glucose) as an alternative reagent for Fehling's solutions. Its advantage is the convenience in having the chemicals combined in a single solution and the fact that the amount of copper reduced by a given amount of sugar is about 10 per cent greater than with Fehling's solution, and the ratio of Cu : glucose more constant. With such a combined

[5] Benedict's qualitative solution may be used for either the cuprous or cupric titration as follows: To 25 cc. of the reagent add sugar solution containing not more than 50 mg. glucose and water to make the total volume 100 cc. Boil 5 minutes and cool. For cuprous titration add 25 cc. iodate-iodide solution, acid, and oxalate, and titrate exactly as described for the use of Fehling's solution. For cupric titration add 5 gm. KI and 25 cc. 5 N H_2SO_4 and titrate. In either case subtract the titration from the corresponding blank. The amount of copper reduced by glucose with this reagent is about 10 per cent less than with Fehling's solution and about 20 per cent less than with our "combined reagent" under the above conditions of heating. (48.8 mg. glucose reduced 90 and 89.8 mg. Cu, or 1.84 Cu:1 glucose.) We have not determined the reduction with other amounts of glucose. The reduction of this reagent by normal urines (5 cc.) is equivalent to 0.05 to 0.10 per cent glucose, although no precipitate is formed.

reagent all that is necessary is to add the sugar solution, boil, cool, acidify, and titrate, and the process is accordingly very simple; but in order to combine all the necessary chemicals in a single solution potassium salts must be used which increase the cost of the reagent.[6] The use of the carbonate reagent for determination of sugar in urine and the extent of its reduction by normal constituents of urine will be treated in a later section of this paper.

Combined Carbonate-Citrate Reagent.

			gm. per liter
0.1	M	copper sulfate (crystalline 5 H_2O)	25.0
0.25	"	potassium citrate (crystalline H_2O)	81.0
0.5	"	" carbonate (anhydrous)	70.0
0.5	"	" oxalate (crystalline H_2O)	92.0
0.1	N I_2	" iodate	3.57
		" iodide	50.0

The citrate, carbonate, and oxalate are dissolved in about 600 cc. of warm water, and into this solution the copper sulfate, dissolved separately, is poured with stirring. The iodate and iodide are together dissolved in 150 to 200 cc. of warm water and added to the alkaline copper solution, which is then cooled and diluted to a liter. This solution is of course to be used only for the cuprous titration. The procedure is as follows.

50 cc. of the reagent are accurately measured into a 300 cc. Erlenmeyer flask to which are added the sugar solution containing not more than 150 mg. of glucose, and water if necessary to make the total volume 100 cc. Cover the flask with a small inverted beaker and place on an asbestos mat over a flame so adjusted as to bring the contents to boiling in 4 minutes. Boil 5 minutes.[7] Cool under running water, add 20 cc. of 5 N H_2SO_4, and

[6] We have estimated the cost of material used in this reagent for a single determination (50 cc.), at present prices, at about 6 cents. The use of sodium carbonate and citrate (in which case the oxalate cannot be incorporated in the reagent, and is added just before titration) reduces the cost by one-third.

[7] The amount of copper reduced by glucose is somewhat greater with this solution than with Fehling's solution. This is in accord with the results of Benedict who pointed out the advantage of the low alkalinity of carbonates in avoiding destruction of glucose. However, the *rate* of the reduction of copper, and presumably of the dissociation of glucose into the

shake (cautiously at first) for a few moments until the cuprous oxide and iodide are completely dissolved. If the solution is slightly warmed (40°) the precipitate dissolves quickly and the solution remains clear; otherwise crystals of cupric oxalate or the deeper blue potassium cupric oxalate may form, but their appearance does not interfere with the end-point. Titrate the solution with 0.1 N thiosulfate.[8] A blank titration on the reagent (50

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hypothetical substance actually oxidized, is slower at lower alkalinity, and the completion of the oxidation requires longer heating than at the alkalinity of Fehling's solution. For this reason it is important to adhere closely to an accepted period of boiling. If this is not done rather irregular results are obtained. Although we have selected 5 minutes of boiling, the reduction is incomplete in this time, and longer heating gives a larger amount of cuprous oxide; though longer heating also causes more reduction by the constituents of normal urine. By prolonging the period of boiling to 10 minutes the reduction is substantially complete and with glucose solutions rather more constant results are obtained, but by so doing the insensitiveness of this reagent to normal urine is largely lost and it possesses little advantage over Fehling's solution.

[8] We find the use of potassium biiodate, $KH(IO_3)_2$, convenient and accurate for the standardization of thiosulfate, and it may also be used instead of iodate in preparing the copper reagents. In the latter case the biiodate solution must be made *alkaline* before mixing with KI, and an *equivalent* amount of the biiodate is used. Molecular weight $KH(IO_3)_2 = 389.95 \times \frac{1}{12} \times \frac{1}{10} = 3.2496$ gm. for 1 liter of 0.1 N I_2 solution when reacting with an excess of KI and acid.

Pure potassium biiodate may be conveniently made as follows: 110 gm. $KClO_3$ are added to 450 cc. warm water containing 40 cc. concentrated HCl in a 2 liter Erlenmeyer flask, and placed in a well ventilated hood. 100 gm. of powdered iodine are added and the mixture is warmed slowly with occasional shaking until the reaction starts. The reaction proceeds rather violently, with the evolution of chlorine, but if not heated too far very little iodine is lost. When the reaction has subsided the solution is boiled for a few minutes and filtered boiling hot by suction. On cooling, about 150 gm. of biiodate crystallizes, which is about 90 per cent pure. This is filtered off by suction and redissolved in about three times its weight of boiling water, the solution being filtered hot. After standing over night (in the ice box) the crop of crystals is filtered by suction and dried (after removing filter paper) in the oven at 100°C. The yield is about 100 gm. or 70 per cent.

In our experience this once recrystallized salt is pure (100.0, 99.96, and 99.95 per cent by analysis; after second recrystallization 99.98 and 99.92; and after a third 99.94 and 99.98 per cent). We are indebted to Mr. M. L. Heidemann for carrying out this preparation and for the analyses, and to Dr. S. W. Clausen for calling our attention to the process which is modified from the work of Lamb, Bray, and Geldard (Lamb, A. B., Bray, W. C., and Geldard, W. J., *J. Am. Chem. Soc.*, 1920, xlii, 1636).

cc. of reagent + 50 cc. of water), after heating as above, is made, and recorded in terms of 0.1 N thiosulfate. From this value the titrations of determinations are subtracted, and the remainder multiplied by 6.36 gives the amount of copper reduced. The ratio of Cu:glucose varies somewhat with the amount of sugar but is more constant than the ratios with Fehling's solution. Chart 1 gives also a curve for this reagent showing the ratios corresponding to varying amounts of copper. The amount of copper found divided by the appropriate ratio gives the amount of sugar determined.

Carbonate-Citrate Reagent for Cupric Titration.—If it is desired to use the carbonate reagent for *either* the cupric or cuprous titrations (or if the potassium salts necessary for the "combined reagent" are not available), a solution of substantially the same reducing power may be made as follows.

	gm. per liter
0.1 M copper sulfate (crystalline)	25.0
0.25 " sodium citrate (crystalline $\frac{1}{2}(11\ H_2O)$)	90.0
0.5 " " carbonate (anhydrous)	50.0

This solution is to be used as above described for the combined reagent, with the following difference. For *cupric* titration add, after cooling, 6 gm. of KI and 25 cc. of 5 N H_2SO_4, titrate with 0.1 N thiosulfate, and subtract the titration from the similarly determined cupric blank for the reagent. For *cuprous* titration, add, after cooling, 25 cc. of the iodate-iodide solution (p. 373), 20 cc. of 5 N H_2SO_4, and 20 cc. saturated potassium oxalate, warm the solution to about 40°, and when the cuprous oxide is all dissolved titrate with thiosulfate and subtract from the blank for the reagent with 25 cc. of the iodate solution.

THE DETERMINATION OF SMALL AMOUNTS OF SUGAR.

For some purposes, such as blood analysis, it is essential to be able to determine a few milligrams or tenths of milligrams of sugar, and in many other circumstances the determination of such small amounts is desirable and convenient. For such conditions the delicate iodometric procedures are admirably suited. We use in such cases a copper-carbonate solution of the following composition.[9]

[9] The composition of this solution as regards copper, tartaric acid, and carbonate is nearly the same as that used by Folin and Wu, and appears to be optimum for copper reduction by dilute sugar solutions. The inclu-

Micro Carbonate-Tartaric Acid Reagent.

Final concentration.		gm. per liter
0.02 M	copper sulfate (crystalline)	5.0
0.05 "	tartaric acid	7.5
0.4 "	sodium carbonate (anhydrous)	40.0
0.02 N I_2	potassium iodide	10.0
	" iodate	0.7
0.1 M	" oxalate	18.4

Dissolve the carbonate in about 400 cc. of warm water, and into this pour with stirring the copper sulfate and tartaric acid dissolved in about 150 cc. of water. Dissolve the iodate, iodide, and oxalate in about 250 cc. of water, rinse into the alkaline copper solution, cool, and dilute to a liter. The above formula is used only for the cuprous titration, but by omitting the iodate, iodide, and oxalate the solution may be used for either method, suitable additions being made previous to titration.

Molecular equivalent amounts of either Rochelle salt (14 gm.) or sodium citrate (16 gm.) may be substituted for the tartaric acid, but in either case the resulting solutions have a lower reducing power, about 10 and 20 per cent less, respectively, than the reagent with tartaric acid, and when such solutions are used it is suggested that a table of standards be prepared by determining 0.25, 0.5, 1, and 2 mg. quantities of pure glucose. When plotted the results should give a straight line, or nearly so, and from the curve intermediate values are obtained by interpolation.

The amount of copper reduced by a given amount of glucose depends not only upon the composition of the solution but upon the conditions of heating and the total volume of the solution. A small amount of glucose is oxidized by the oxygen dissolved in the solution and some reoxidation of cuprous oxide takes place especially during the heating. Somewhat greater reduction is

sion of iodate and iodide in this solution as in the combined macro reagent, follows the suggestion of Maclean. Their presence in the alkaline solution has no effect during the oxidation of the sugar, but has the great advantage, over the procedure described by Scales for instance, that the iodine formed on acidification at once oxidizes the cuprous salt as rapidly as the latter is formed by solution of cuprous oxide and thus avoids all danger of its oxidation by air. It will be noted that in using Fehling's solution as above described the iodate solution is added before the mixture is acidified, for the reason just stated.

obtained by heating in an atmosphere of illuminating gas, but the extent of oxidation of sugar and cuprous oxide by air is practically constant as proved by agreement of duplicate determinations under the same conditions, and, by being included in the standard determinations on which the tables are based, does not introduce error. The standard values must, however, be determined under definite conditions as to volume of liquid, duration and manner of heating, and as to size and shape of vessel containing the solution.

The following series of determinations show the effect of varying the conditions of heating, especially the effect of exposure to air during and after the heating.

5 cc. of the reagent and 5 cc. of solution containing 0.5 or 1.0 mg. of glucose were boiled for 2 minutes in large test-tubes over a microburner. These results show that the greatest error is due to oxidation of sugar or of cuprous oxide *during the heating*, and that when the tubes are covered so as to minimize convection currents the amount of reduction is reasonably constant and the error small. When cold the reoxidation by air is slow.

	Copper reduced.	
	1.0 mg. glucose.	0.5 mg. glucose.
	mg.	*mg.*
Test-tube covered.		
Titrated at once when cold	1.97 2.02 2.05 2.00	1.01 1.04 0.98 0.96
Titrated 20 min. after cooling		1.01 1.04
After cooling, aerated 2 min		0.95
Before boiling, added 5 cc. water, total volume 15 cc.	1.95 1.95	0.82
Heated reagent to boiling, then added glucose	2.02 2.00	0.98 1.01
Not covered during boiling.		
Aerated *during* boiling	1.71	0.78 0.59

3

TABLE II.

Amounts of Glucose Corresponding to Copper. Micro Reagent. Cuprous Titration. 5 Cc. Reagent + 5 Cc. Sugar Solution.

Cu reduced.	Microburner 2½ min.		Water bath 15 min.		0.005 N iodine required to oxidize cuprous oxide.	Glucose in blood.*	
	Glucose.	Glucose in blood.*	Glucose.	Glucose in blood.*		Microburner 2½ min.	Water bath 15 min.
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
mg.	*mg.*	*per cent*	*mg.*	*per cent*	*cc.*	*per cent*	*per cent*
0.1	0.07	0.014	0.07	0.014	0.5	0.020	0.020
0.2	0.133	0.026	0.13	0.026	1.0	0.040	0.040
0.3	0.186	0.037	0.18	0.037	1.5	0.056	0.056
0.4	0.235	0.047	0.24	0.047	2.0	0.067	0.067
0.5	0.278	0.055	0.29	0.058	2.5	0.082	0.083
0.6	0.326	0.065	0.33	0.066	3.0	0.100	0.096
0.7	0.374	0.075	0.37	0.074	3.5	0.114	0.109
0.8	0.415	0.083	0.41	0.082	4.0	0.130	0.122
0.9	0.465	0.093	0.45	0.090	4.5	0.145	0.135
1.0	0.515	0.103	0.50	0.100	5.0	0.160	0.148
1.1	0.564	0.113	0.54	0.108	5.5	0.175	0.162
1.2	0.614	0.123	0.58	0.116	6.0	0.189	0.176
1.3	0.664	0.133	0.62	0.124	6.5	0.202	0.188
1.4	0.711	0.142	0.66	0.132	7.0	0.217	0.200
1.5	0.758	0.151	0.70	0.140	7.5	0.232	0.214
1.6	0.804	0.161	0.75	0.150	8.0	0.245	0.227
1.7	0.852	0.170	0.79	0.158	8.5	0.259	0.243
1.8	0.90	0.180	0.83	0.166	9.0	0.274	0.255
1.9	0.945	0.189	0.88	0.176	9.5	0.287	0.268
2.0	0.99	0.198	0.91	0.182	10.0	0.300	0.280
2.2	1.077	0.215	1.00	0.200	10.5	0.315	0.292
2.4	1.17	0.234	1.08	0.216	11.0	0.332	0.305
2.6	1.255	0.251	1.17	0.234	11.5	0.349	0.317
2.8	1.34	0.268	1.25	0.250	12.0	0.365	0.329
3.0	1.428	0.285	1.33	0.266	12.5	0.380	0.342
3.2	1.515	0.303	1.42	0.284	13.0	0.396	0.356
3.4	1.61	0.322	1.49	0.298	13.5	0.413	0.369
3.6	1.714	0.343	1.56	0.312	14.0	0.430	0.383
3.8	1.815	0.363	1.63	0.326	14.5	0.449	0.397
4.0	1.914	0.383	1.71	0.342	15.0	0.457	0.410
4.2	2.02	0.404	1.80	0.360			
4.4	2.12	0.424	1.89	0.378			
4.6	2.24	0.448	1.98	0.396			
4.8			2.06	0.412			

* Figures in Columns 3, 5, 7, and 8 are used only when 5 cc. of 1:10 blood filtrate, equivalent to 0.5 cc. of blood, are taken for the determination. They apply also for urine containing small amounts of sugar, when 5 cc. of 1:10 diluted urine or urine filtrate are taken. For urine containing more than 0.4 per cent sugar, which must be diluted 1:50, 1:100, or 1:200, and 5 cc. of the dilution are used, the values in these columns are to be multiplied by 5, 10, or 20, respectively.

TABLE III.

Amounts of Glucose Corresponding to Copper. Micro Reagent. Cuprous Titration.

(1) 2 cc. reagent + 2 cc. sugar solution heated 10 min. in boiling water bath.			(2) 10 cc. reagent + 10 cc. sugar solution heated 20 min. in boiling water bath.		
Copper reduced.	Glucose.	Glucose in blood.	Copper reduced.	Glucose.	Glucose in blood.
mg.	*mg.*	*per cent*	*mg.*	*mg.*	*per cent*
0.1	0.083	0.042	0.4	0.38	0.038
0.2	0.124	0.062	0.6	0.47	0.047
0.3	0.165	0.083	0.8	0.52	0.052
0.4	0.206	0.103	1.0	0.62	0.062
0.5	0.247	0.124	1.5	0.84	0.084
0.6	0.288	0.144	2.0	1.03	0.103
0.7	0.329	0.165	2.5	1.25	0.125
0.8	0.370	0.185	3.0	1.45	0.145
0.9	0.410	0.205	3.5	1.66	0.166
1.0	0.452	0.226	4.0	1.87	0.187
1.1	0.493	0.247	4.5	2.07	0.207
1.2	0.534	0.267	5.0	2.27	0.227
1.3	0.575	0.288	5.5	2.48	0.248
1.4	0.614	0.307	6.0	2.69	0.269
1.5	0.656	0.328	6.5	2.90	0.290
1.6	0.698	0.349	7.0	3.10	0.310
1.7	0.739	0.370	7.5	3.30	0.333
1.8	0.780	0.390	8.0	3.50	0.350
1.9	0.821	0.411	9.0	3.92	0.392
2.0	0.862	0.431	10.0	4.32	0.432

We have determined the degree of reduction with the tartaric acid solution with varying amounts of glucose under the following conditions, the heating being carried out in all cases in Pyrex test-tubes, 20 × 200 mm., covered with small inverted beakers or bottle caps.

2 cc. reagent + 2 " sugar solution } ..... 10 min. in boiling water bath.

5 " reagent + 5 " sugar solution } ..... 15 " " " " " "
..... 2½ " boiling over microburner.

10 " reagent + 10 " sugar solution } ..... 20 " in boiling water bath.

From a number of series of careful determinations on solutions of United States Bureau of Standards glucose, the standard values given in Tables II and III have been calculated. We

find it most convenient to use 5 cc. of the reagent for a determination and for this volume Table II contains detailed data for the two methods of heating, and for the cuprous titration.

Procedure.—Measure 5 cc. of the reagent into a large test-tube, and add an equal volume of the sugar solution containing not more than 2.0 mg. of glucose. Cover the tube and either boil for 2½ minutes over a small flame or place in a boiling water bath for 15 minutes. Cool for several minutes under the tap. If using the combined reagent containing oxalate and iodate, add 5 cc. of N H_2SO_4 and after about 1 minute titrate with 0.005 N thiosulfate. A blank titration on the reagent is determined after heating with an equal volume of water. The value of the blank does not change during at least several months and therefore need be determined only occasionally. The difference between the blank and the titration of a determination represents reduced copper, and the corresponding amount of sugar is learned by inspection of the table (1 cc. 0.005 N thiosulfate = 0.318 mg. copper). When 10 or 2 cc. of the reagent are used a proportionate amount of acid is used for acidification, and the corresponding table is used for the calculation.

The following results of a series of determinations of copper reduced by known amounts of pure glucose, using 10 cc. of reagent (not containing oxalate) + 10 cc. of sugar solution, and heated 20 minutes in a boiling water bath, show the close agreement of the cupric and cuprous titrations and are illustrations of the data from which the standard tables and curves are derived.

Glucose taken.	Copper reduced.	
	Cuprous titration.	Cupric titration.
mg.	*mg.*	*mg.*
0.4	0.47	0.46
0.8	1.40	1.40
1.2	2.34	2.34
1.6	3.32	3.38
2.0	4.30	4.24
3.0	6.88	6.92
4.0	9.42	9.50
5.0	11.61	11.68

The Determination of Sugar in Milk, Urine, and Blood.

Milk.

Precipitate the protein and fat by tungstic acid as proposed by Folin and Wu (14) for blood. 10 cc. of milk and 80 cc. of water are measured into a 200 cc. flask. Add 5 cc. of 10 per cent sodium tungstate and 5 cc. of $\frac{2}{3}$ N H_2SO_4. Shake well and pour through a folded filter. The filtrate should be perfectly clear. 25 or 50 cc. of the filtrate, equivalent to 2.5 or 5.0 cc. of milk, are added to 25 cc. of each of the Fehling's Soxhlet solutions (25 cc. of water are also added if 25 cc. of filtrate are used), and the determination is carried out as above described, using either the cuprous or the cupric titration, the former being somewhat preferable. Consult Munson-Walker tables (15) for the conversion of copper into terms of lactose. Instead of the Munson-Walker tables the lactose curve of Chart 1 may be conveniently used. This and the glucose curve have been prepared from their tables and give the ratio of copper: sugar with varying amounts of copper. The amount of copper found divided by the corresponding ratio gives the amount of sugar.

Urine.

The Fehling's Soxhlet solutions may be used as above described also for the determination of sugar in urine, using 5 cc. of urine + 45 cc. of water, though the results so obtained are too high because of the reduction of copper by constituents other than sugar. Normal urine, of specific gravity 1.020 and giving a negative test with Benedict's qualitative reagent, gives a reduction corresponding to 0.18 to 0.25 per cent of glucose, though no precipitation of cuprous oxide occurs. This normal reduction which is only slightly decreased, if at all, after fermentation is equivalent to a plus error of about 2 to 4 gm. of glucose in a 24 hour urine. With urines containing much glucose, this error, although considerable, is in many cases not important. The procedure is convenient in that no preliminary dilution of the urine is necessary unless it contains over 5 per cent of sugar. But for urines containing only little sugar, or when greater accuracy is desired, the *direct* use of Fehling's solution is not to be recommended.

With the carbonate reagents the error due to reduction by normal constituents is less, but still considerable. With 5 or 10 cc. of urine, diluted to 50 cc., with 50 cc. of the combined carbonate-citrate reagent (p. 375), a series of ten different normal urines, giving negative qualitative tests with Benedict's reagent, gave results equivalent to from 0.07 to 0.14 per cent of glucose, or about half the values obtained with Fehling's solutions. With the micro carbonate reagent, 5 cc. of normal urines previously diluted 1:10 (0.5 cc. of urine) give about the same values. In neither case is the apparent amount of sugar in such normal urines very materially decreased by fermentation, which shows that the reduction with the carbonate reagents also is due in great part to substances other than glucose. Except for urines containing very small amounts of glucose, when the percentage error is larger, and when the preliminary precipitation to be described later should be used if more accurate results are desired, the error caused by the normal reduction with the carbonate reagents may perhaps be disregarded. When the amount of sugar present is such that preliminary dilution of the urine is necessary, the concentration of creatinine and other interfering substances is thereby reduced to a point at which they no longer cause reduction and the small error due to their presence quite disappears. When used as stated below, neither of the carbonate reagents is reduced by such amounts of protein, acetone, acetoacetic acid, or chloroform as may occur in urine; nor do these substances or the normal constituents of urine interfere with the iodometric titrations.

With the above statement of limitations, the application to urine of the methods here described may be summarized. From a qualitative test judge whether the amount of sugar present is large or small, and use one of three alternative methods. Because of its simplicity and quickness we commonly use the micro method with the "combined reagent" and the cuprous titration.

Fehling's Solution.—Use 5 cc. urine + 45 cc. water with 25 cc. each of Fehling's Soxhlet solutions as described on page 371. Applicable with urines containing up to 5 per cent sugar; if known to be very high, use 2 cc. or less urine.

Macro Carbonate-Citrate Reagent.—Use 5 cc. urine + 45 cc. water with 50 cc. of reagent as described on page 375. Applicable up to 2 per cent sugar; if strong qualitative test, use 1 or 2 cc. urine, or 5 cc. previously diluted 1:2 or 1:5.

Micro Reagent.—Dilute the urine 1:20, 1:50, or 1:100, as indicated by the qualitative test, and use 5 cc. with 5 cc. of reagent as described on page 382.

If the qualitative test is only faintly positive the preliminary treatment described below should be followed.

The Reduction of Normal Urine and the Determination of Small Amounts of Sugar in Urine.

With urines containing only a few tenths of a per cent of sugar, no one of the existing methods gives, according to our experience, even approximately correct results without preliminary removal of the interfering substances. Of the methods used for such removal, those based on precipitation by mercuric salts appear to be the most satisfactory and have been most often used. While such methods perhaps are an outgrowth of the older urea method of Liebig, the first to use mercuric salts preliminary to sugar determination appears to have been Johnson (16) who in 1887 used $HgCl_2$ to remove creatinine previous to the determination of sugar in urine by the picric acid colorimetric method which he says had been introduced in 1883 by George Johnson. Patein and Dufau (17) in 1902 described the preparation and use of a solution of acid mercuric nitrate for the treatment of urine and other solutions preliminary to sugar determination. Their formula has been repeatedly used (18) and is probably the origin of the solution recently described by Benedict and Osterberg (19). The procedure of Patein and Dufau, improved by the substitution of sodium bicarbonate for sodium hydroxide, proposed by Benedict, is an almost ideal preparation for glucose determination. It removes nearly all interfering substances, and does not precipitate, adsorb, or destroy glucose. It does, however, leave some mercuric salt in the filtrate, and as pointed out by Patein and Dufau this must be removed before treatment with alkaline copper solution. It is not clear why this should be necessary for the cuprous iodometric titration, which we expected would measure the total reduction, both copper and mercury, but the presence of mercury salts does cause slightly low results.[10]

[10] With pure glucose solutions, the filtrates contain only the merest trace of mercury and correct results are obtained without subsequent treatment with sulfide and copper, while with glucose added to urine the results are low unless the mercury, present in much greater amount, is removed.

The mercury is removed from the acidified filtrate by Denigès (18), Schöndorff (20), and Benedict and Osterberg (19) by the use of Zn or Zn-Cu. This plan is not permissible for the iodometric methods because of the reduction of nitrate to nitrite, the latter liberating iodine from KI on later acidification. We remove the mercury from the filtrate by a small excess of $(NH_4)_2S$ or Na_2S, the excess of sulfide being in turn removed by $CuSO_4$, the excess of which remaining in the filtrate is with the cuprous titration not objectionable. Treated in this way, the details being presented below, urines yield filtrates which behave altogether like sugar solutions, and may be analyzed with as great accuracy. Added glucose is recovered quantitatively.

The filtrates from normal urines contain according to our method surprisingly little reducing substance, commonly the equivalent of about 0.02 or 0.03 per cent of glucose in the urine. Very little if any of this reducing substance is as a rule fermented by yeast, which indicates that it is not glucose; and from this fact it follows that at least many normal urines actually contain practically no glucose. We shall consider in a later paper the extent of the variations in the amount of the reducing substance of the urine of normal subjects; but it may be noted here that our results are similar to but by no means identical with those recently reported by Benedict, Osterberg, and Neuwirth (21) who found the reduction of normal urine to be equivalent to from 0.05 to 0.10 per cent or more of glucose, *about one-third of which was fermentable by yeast.* The results by our technique indicate somewhat smaller amounts of reducing substance, and that *little if any of it is, as a rule, fermented by yeast.*

The solutions used for the mercury precipitation and subsequent treatment are: the Patein-Dufau solution of acid mercuric nitrate, prepared in the manner recently stated by Benedict and Osterberg (19); a strong solution of Na_2S or $(NH_4)_2S$, free from sulfites;[11] and a strong solution of $CuSO_4$. The precipitation is

[11] Sulfites (or thiosulfate), if added with the sulfide, are not removed by the later treatment and on acidifying are oxidized by and use up iodine, giving too high results. On adding an excess of $ZnSO_4$ to a few cc. of the sulfide solution, the filtrate when acidified and when starch is added should give a blue color with the first or second drop of iodine. A satisfactory solution of Na_2S may be made by passing washed H_2S from a generator into NaOH.

TABLE IV.

Reduction of Normal Urine, and Normal Urine with Added Glucose.

Normal urines.	Final dilution of filtrate.	Blank minus titration.	Copper reduced.	Glucose found in urine.		Average.	After fermentation.
		cc. 0.005 N I	*mg.*	*mg.*	*per cent*	*per cent*	*per cent*
I	1:4	1.60	0.508	0.28	0.022		
	1:4	1.35	0.43	0.25	0.020		
	1:4	1.40	0.445	0.254	0.020	0.021	0.018
Same + 0.04 per cent glucose.	1.4	4.5	1.43	0.73	0.058		
	1:4	4.6	1.46	0.74	0.059		
	1:4	5.1	1.62	0.814	0.065		
	1:4	5.2	1.65	0.828	0.066	0.062	0.019
II	1:2	3.2	1.02	0.52	0.021		
	1:5	1.2	0.38	0.23	0.023		
	1:5	1.2	0.38	0.23	0.023	0.022	
III	1:5	0.45	0.14	0.09	0.009		
		0.40	0.13	0.09	0.009	0.009	0.012
Same + 0.10 per cent glucose.	1:5	7.3	2.32	1.14	0.114		
	1:5	7.2	2.29	1.12	0.112	0.113	0.012
IV (decomposed).	1:5	0.2	0.06				
Same + 0.100 per cent glucose.	1:5	6.5	2.07				
	1:5	6.6	2.10				
			Average 2.08				
			Minus 0.06				
			2.02	1.00	0.100		
V	1:2	4.7	1.50	0.75	0.030		0.036
Same by Benedict method.					0.046		
VI	1:2				0.035		0.037
VII (after eating apples).	1:5	5.2	1.65	0.83	0.083		0.084

carried out substantially as described by Benedict and Osterberg, by the use of equal volumes of urine and mercuric nitrate (25 cc.) and by the addition of solid $NaHCO_3$ to slight alkaline reaction to litmus paper. A drop of caprylic alcohol lessens frothing and introduces no error. If the urine is known to contain sugar it may be diluted with a known amount of water before precipitation; even "normal" urines may be diluted so that the filtrate is 1:5 instead of 1:2 and so allow more rapid filtration. The filtrate, 15 cc. or more, is faintly acidified by a few drops of concentrated H_2SO_4, a few drops of Na_2S (excess), and, after shaking, a few drops (excess) of $CuSO_4$ are added. The sulfides of mercury and copper are filtered off, leaving a water-clear filtrate which should contain a very small amount of copper. If acid, it is quite permanent and keeps indefinitely without change in its reducing power, but if slightly alkaline a faint precipitate of cuprous hydroxide may appear after a day or so. Unless the filtrate is to be analyzed the same day, it should be made faintly acid, if not already so. 2, 5, or 10 cc. of this filtrate are heated with an equal volume of the combined micro reagent as already described, using the cuprous titration (p. 382). The results in Table IV illustrate the amount of copper reduced by filtrates of normal urines and show the degree of accuracy in the determination of added glucose by the method here described.

Blood.

For the preparation of the blood filtrates we employ the Folin-Wu (14) tungstic acid precipitation, since such filtrates are used also for the determination of other constituents. But coagulation of protein by heat and colloidal iron is also satisfactory.

To a measured quantity of oxalate blood (2 to 15 cc., 2 or even 1 cc. being sufficient for the sugar determination) add seven volumes of water and allow to lake. Add one volume of 10 per cent sodium tungstate and one volume of $\frac{2}{3}$ N H_2SO_4. Stopper and shake; filter after 5 minutes. 2, 5, or 10 cc. of this filtrate, equivalent to one-tenth the volume of blood, are used with an equivalent volume of the micro reagent as described on page 382.

These directions are applicable for all bloods containing not more than 0.4 per cent of glucose; for higher concentrations, the

blood filtrates are diluted or a smaller amount (and water) is taken for the determination.

A comparison of the results by this and other methods will be reported by one of us in a later paper. It may be stated, however, that results by our method are in general substantially the same as by the methods of Folin and Wu, Maclean (8), and usually by the Myers-Bailey modification of the Lewis-Benedict method. All these methods in our hands not infrequently yield results which are much lower than the last procedure described by Benedict (22), and higher than the method described by Shaffer (23). According to our experience the technique here described is more reliable as well as more convenient than any of the other methods above mentioned.

SUMMARY.

1. The reversible reaction

$$Cu^{++} + I^- \rightleftarrows Cu^+ + I_2$$

is applied to the determination of the mixture of cuprous and residual cupric copper resulting from the action of reducing sugar upon alkaline copper solutions. The reaction may be caused to take place to completion *in either direction*, thus enabling one to determine by iodometric titration either the cupric or the cuprous copper in a mixture of the two forms.

2. The details of methods are given for both cupric and cuprous titrations, the latter being generally preferred. The use of several reagents for the determination of glucose by means of the cupric and cuprous titrations is described.

3. The methods are applied to the determination of reducing sugar in milk, blood, and urine, as well as in other solutions.

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